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Physiological and molecular characterisation of 'stay green' mutants in durum wheat.

Physiological and molecular characterisation of 'stay green' mutants in durum wheat

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A thesis submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Science.

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Declaration

The work reported in this dissertation was carried out by the author in the Department of Agricultural Science, University of Bristol, UK.

The dissertation has not been submitted, in whole or in part, for any other degree at this or any other University. The views expressed here are those of the author and not of the University of Bristol.

Giuseppe Spano

DEDICATA…..

*A **mio Padre,***

*A **mia Madre, a Laura** e **Tiziana** che hanno potuto vedermi, sentirmi e sostenermi,*

*A **mio zio** e ai nostri sogni rubati,*

*Al genio e all'entusiasmo del mio Direttore, **Dr Natale Di Fonzo,***

*A **Carla** che ha creduto, spezzato, sollevato e seguito,*

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LIST OF ABBREVIATIONS

The abbreviations given below are used throughout Chapters 1 to 4. The list does not include

- Atomic and chemical symbols

3'	3 prime (COOH) terminus of DNA
5'	5 prime (phosphate) terminus of DNA
%	Per cent
°C	Degrees Celsius
µg	Microgramme
µg μl^{-1}	Microgramme *per* microlitre
µg ml^{-1}	Microgramme *per* millilitre
µl	Microlitre
µl L^{-1}	Microlitre *per* litre
µm	Micron
µM	Micromolar
µmol mol^{-1}	Micromole *per* mole
µmol m^{-2} s^{-1}	Micromole *per* meter square *per* second
µmol photons m^{-2} s^{-1}	Micromole *per* photons meter square *per* second
A	Adenine
A_{260}	Spectrophotometric absorbance at 260 nanometer
A_{280}	Spectrophotometric absorbance at 280 nanometer
A_{663}	Spectrophotometric absorbance at 663 nanometer
A_{646}	Spectrophotometric absorbance at 646 nanometer
ABA	Abscisic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
A. thaliana	*Arabidopsis thaliana*

bp	Base pair
C	Cytosine
CAB	Chlorophyll a/b binding protein gene
cDNA	Complementary deoxyribonucleic acid
Chl	Chlorophyll
Ci	Leaf internal CO_2
cm	Centimetre
Cys	Cysteine
CIMMYT	International Centre for Maize and Wheat Improvement
cv	Cultivated variety (cultivar)
DAF	Days after flowering
DCH	Differential colony hybridisation
DDRT-PCR chain reaction	Differential display reverse transcription-polymerase
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
dT	Deoxythymidine
DTT	Dithiothreitol
EB	Ethidium bromide
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethanesulphonate
Fig.	Figure
G	Guanine
g	Gramme
*x*g	accelleration of gravity
g m^{-2}	Gramme per meter square

h	Hour
IACR	Institute of Arable Crops Research
kb	Kilobase
Kg	Kilogramme
LAI	Leaf area index
LARS	Long Ashton Research Station
LB	Luria-Bertani medium
LBA	Luria-Bertani medium supplemented with ampicillin
LBK	Luria-Bertani medium supplemented with kanamicin
LBT	Luria-Bertani medium supplemented with tetracyclin
Lys	Lysine
M	Molar
m^3	Cubic meter
mg	Milligramme
mg ml^{-1}	Milligramme *per* millilitre
min	Minute
ml	Millilitre
ml l^{-1}	Millilitre *per* litre
mm	Millimetre
mm^2	Millimetre square
mM	Millimolar
mg g^{-1}	Milligramme *per* gramme
mRNA	Messager ribonucleic acid
M_2	Second generation progeny line of a primary mutagenised lines
M_5	Sixth generation progeny line of a primary mutagenised lines
NAD	Nicotinammideadenindinucleotide
NADH	Nicotinammideadenindinucleotide reduced

nm	Nanometer
OD	Optical Density
PAR	Photosynthetically active radiation
PCD	Programmed cell death
PCR	Polymerase chain reaction
Pn	Net photosynthesis
pfu	plaque forming units
QTL	Quantitative trait loci
RNA	Ribonucleic acid
rpm	Revolutions *per* minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RuBP	Ribulose-1,5-bisphosphate
s	Second
SAGs	Senecence associated genes
SDGs	Senescence down-regulated genes
SDW	Sterile distilled water
SE-HPLC	Size exclusion high performance liquid chromatograph
SH	Subtractive hybridisation
sid	Senescence induced degradation
SSU	Rubisco small subunit gene
T	Thymine
TBE	Tris-borate buffer
TE	Buffere consisting of Tris-HCL and EDTA
U	Unit
U μl^{-1}	Unit per microlitre
UV	Ultraviolet

v/v	volume *per* volume
W	Watt
WT	Wild type
w/v	weight *per* volume

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"Stay green phenotype in durum wheat: is it time to increase the sink?
SEB meeting, Heriot-Watt University, Edinburgh, J. Exp. Bot., March 1999.

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Biotechnology of Cereals: Tools, Targets and Triumphs., 16th IACR Long Ashton International Symposium (Long Ashton, Bristol, UK) 13-15 September 1999).

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'Stay green' mutants in durum wheat.
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Abstract

About 20.000 seeds of *Triticum durum* desf. (cv Trinakria) were treated either with EMS (10.000) or with Sodium Azide (10.000) and four M_5 progeny plants grown in the field (Foggia, Italy) were chosen for their similar timing of flowering, but later timing of senescence.

Physiological characterisation was carried out in a controlled-environment glasshouse in IACR-Rothamsted, Harpenden, UK. Net photosynthesis, chlorophyll concentration, leaf internal CO_2 (Ci), stomatal conductance and the fluorescence of chlorophyll "a" were measured on attached flag leaves at weekly intervals from flowering until full senescence.

Total nitrogen, the nitrogen distribution in protein fractions and HPLC analyses of proteins were also performed on flour milled from randomly selected grains.

Differential display reverse transcription–polymerase chain reaction (**DDRT-PCR**) was used as molecular technique to identify genes that were differentially expressed in the mutant and control plants. Different stages of leaf development and combinations of probes were used and the fragments identified were screened for differential expression.

The results presented show that an extension of photosynthesis could have importance for increasing yield in durum wheat.

Chapter 1

General Introduction

1.1. Photosynthesis

The average global grain yield per unit area of the major cereal crops, wheat, rice and maize, has more than doubled over the period between 1940 and 1980 and this trend continues (Evans, 1993). This doubling of grain yield has coincided with the period when our understanding of photosynthesis has increased greatly.

The synthesis of organic compounds from inorganic precursors requires energy and reducing power (low-potential electrons). The overall process whereby plants, algae and prokaryotes directly use light energy to synthesise organic compounds is called photosynthesis. Photosynthesis is the process by which solar energy is captured and converted into chemical energy stored in the form of carbohydrate. This process takes place in the presence of chlorophyll, the green pigment in plant leaves, and requires light energy to drive the synthetic process.

The overall process of photosynthesis in green leaves can be simplified as follows:

$$CO_2 + 2H_2O \xrightarrow[\text{Chloroplasts}]{\text{Sunlight}} (CH_2O) + O_2 + H_2O$$

[where **(CH_2O)** is one–sixth of a glucose molecule] and it may be generalised as the process that converts sunlight into crop yield as shown in **Fig.1.**

As shown in the equation, photosynthesis is a biological **oxidation-reduction** (or **redox**) process. CO_2 is the electron acceptor and H_2O serve as the electron donor. (CH_2O) represents the carbohydrate generated by the reduction, and O_2 represents the product formed by oxidation of H_2O.

1.2. Photosynthesis and productivity

The process of photosynthesis is pivotal to the production of food and fibre as it provides the raw materials for all plant products. Historically, the goal of increasing crop productivity has been achieved by repeated selection and cross–breeding of the most productive strains, and breeders have been very successful in raising the genetic yield potential of cereals such as wheat and maize (**Fig. 1.1 A** and **B**).

This success has been determined, largely, by genetic exploration of variation in the partition of photosynthate to the harvested product (i.e. harvest index) (Nelson, 1988). Recently, the advent of genetic manipulation (GM) technology has offered the possibility of increasing carbon assimilation more rapidly through genetic engineering rather than by selective breeding. However, although the mechanisms of carbon assimilation are well understood and the techniques to modify them are established, our greater understanding of the photosynthesis has not yet contributed to yield increases.

Although photosynthesis is fundamental to plant productivity, many other factors modify the magnitude of productivity attained in the field. In order to identify selectable traits in breeding programme to improve crop photosynthesis it is important to consider the components of biomass production.

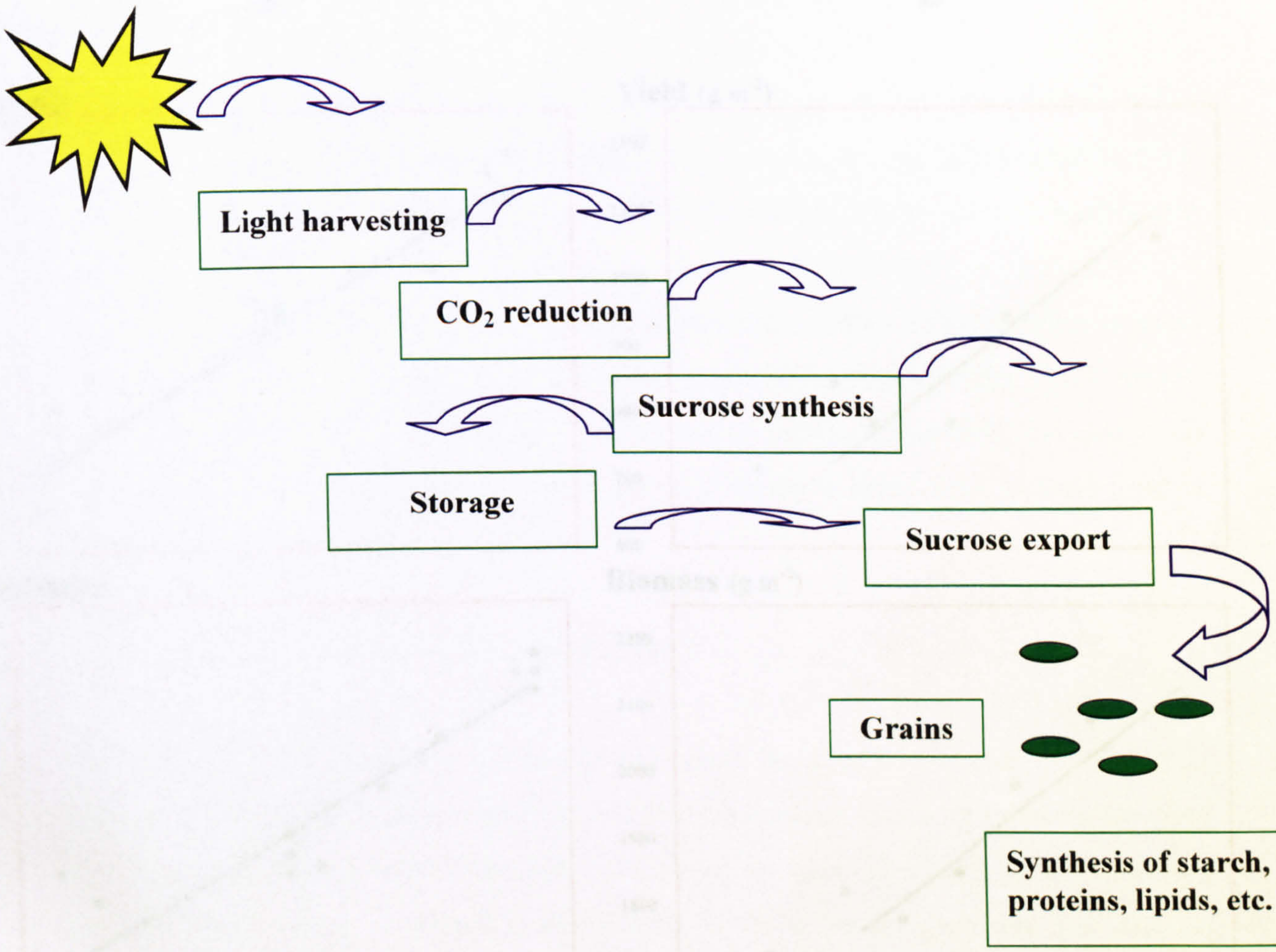


Fig.1. Photosynthesis from light harvesting to grain production.

The flow diagram indicates the principal subsystems that convert sunlight into crop yield.

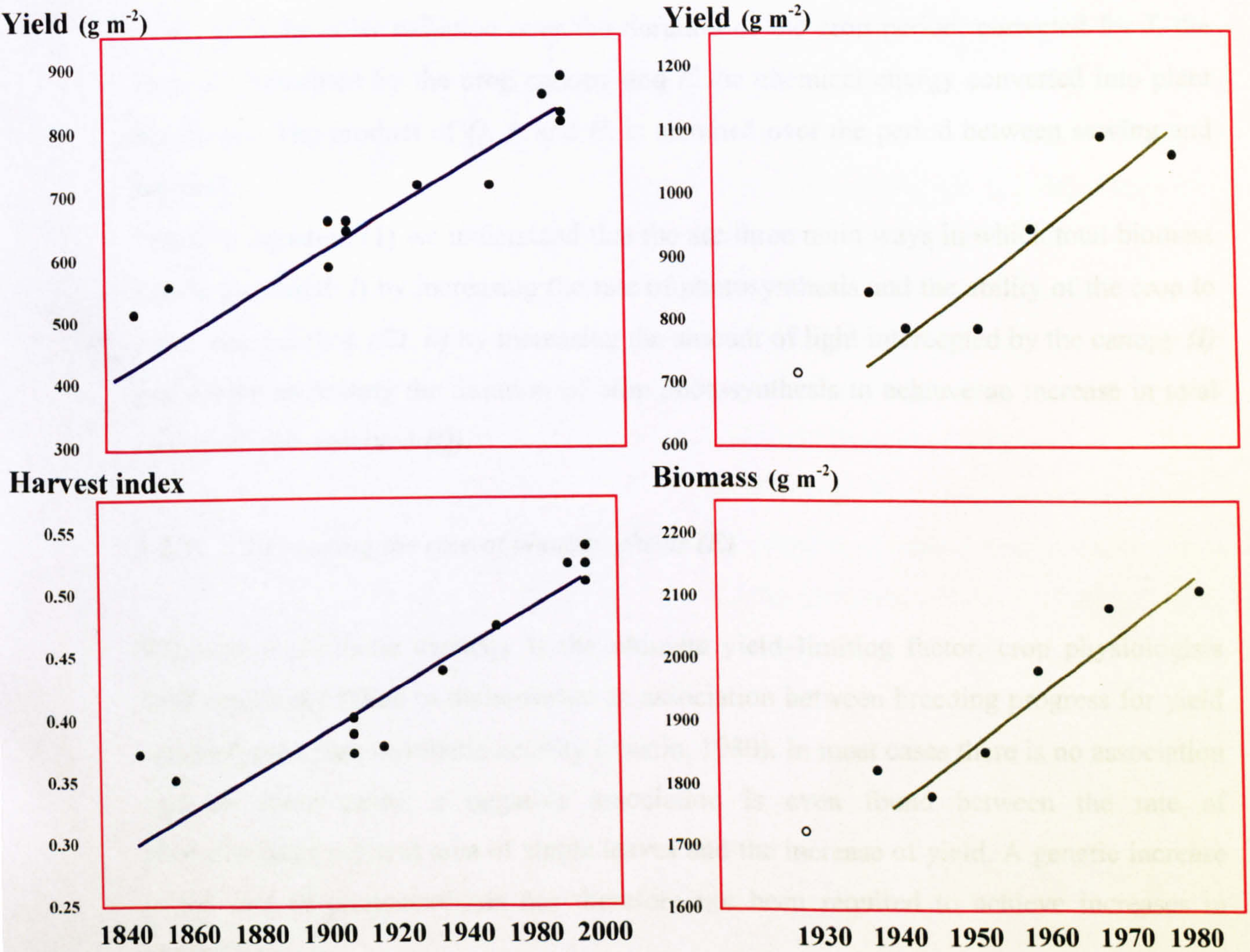


Fig.1.1. **A)** Relationship between grain yield and harvest index of wheat cultivars since 1820. **B)** Relationship between grain yield and biomass of an open–pollinated (○) variety and hybrids (●) of maize from 1930 to 1980.

The increase in wheat has resulted from an increase in harvest index (**A**), whereas the increase in maize is largely associated with an increase in above–ground biomass (**B**) (adapted from Richards, 2000)

Without water limitation, biomass production can be expressed as:

$$\textbf{Biomass} = \sum_{\textbf{sowing}}^{\textbf{harvest}} Q \times I \times E \qquad (1)$$

where Q is the solar radiation over the duration of the crop period, corrected for I, the amount intercepted by the crop canopy and E the chemical energy converted into plant dry matter. The product of Q, I, and E, is summed over the period between **sowing** and **harvest.**

From the equation **(1)** we understand that the are three main ways in which total biomass can be increased: ***i)*** by increasing the rate of photosynthesis and the ability of the crop to retain fixed carbon ***(E)***, ***ii)*** by increasing the amount of light intercepted by the canopy ***(I)*** and ***iii)*** by increasing the duration of crop photosynthesis to achieve an increase in total solar radiation received ***(Q).***

1.2.1. *Increasing the rate of photosynthesis (E)*

While photosynthetic capacity is the ultimate yield–limiting factor, crop physiologists have repeatedly failed to demonstrate an association between breeding progress for yield and increased photosynthetic activity (Austin, 1980). In most cases there is no association and, in some cases, a negative association is even found between the rate of photosynthesis per unit area of single leaves and the increase of yield. A genetic increase in the rate of photosynthesis has therefore not been required to achieve increases in productivity.

Genetic differences in the rate of leaf photosynthesis are often reported but such differences are rarely associated with higher productivity (Evans, 1993), and, even when selection programmes have raised leaf photosynthesis by 8 to 15%, no yield increase has occurred (Nelson, 1988).

Different species have been evaluated for photosynthesis:yield relationships, with photosynthesis being measured on a leaf area basis. Comparisons between old and new cultivars of many species, including wheat, rice, sorghum, and soybean, show that yield improvement has been associated with a decline, rather than increase, in the rate of the photosynthesis, relatively to that of their progenitors.

However, the absence of any relationship between rate of photosynthesis per unit area of single leaves and an increase of yield cannot be taken as dogma. Some evidence suggested that such an association might exist for modern semi-dwarf wheat. Reynolds *et al.* (2000) showed that an increase in photosynthetic rate in modern spring wheat varieties was associated with yield improvement, although it **appeared to be largely due to a delayed senescence during grain filling.** Fischer *et al.* (1996) have analysed several lines produced at CYMMYT (International Centre for Maize and Wheat Improvement) in Mexico, and found that both stomatal conductance and maximum photosynthesis rate were associated with yield increase. **However, among the photosynthetic characteristics, the correlation of yield with leaf conductance was stronger.**

Other evidence suggests that breeding for high seed yield has led to improvement of photosynthesis in soybean (Buttery *et al.* 1981), **but selection for high leaf photosynthesis did not increase seed yield in soybean** (Ford *et al.* 1983).

Since genetic changes in the rate of leaf photosynthesis have not accompanied yield increases in the major crops, it is probable that leaf photosynthesis does not limit grain yield, but that other factors might be limiting. The identification of these limiting factors, followed by selection to remove the limitation, may result in the incorporation of new genetic variation into breeding programmes.

In conclusion there is no clear evidence that total biomass would be increased by genetic increase in the rate of photosynthesis

1.2.2. *Increasing the duration of crop photosynthesis (Q)*

A longer duration of crop photosynthesis offers an opportunity of increasing the total amount of carbon fixed, by simply extending the solar radiation **(Q)** available during the entire growth period. For example, in field-grown rice, biomass increased by 0.2 t ha^{-1} for each day that growth duration was extended (Akita, 1989). However, is not just the duration of growth that can be manipulated to increase biomass and yield, but also its timing. Therefore, manipulating crop phenology in order to have a better match periods of high radiation with a critical growth stages can be important. Timing may also be important to increase yield and biomass in relation to water supply. If water is a major

limitation then maximising growth when conditions are cool and vapour pressure deficit is low will increase water use efficiency and biomass production (Richards, 1991). Apparently, extending crop duration is the simplest genetic way to increase crop biomass and yield.

1.2.3. *Improving light interception by the leaves (I)*

Since the capacity for photosynthesis is dependent on the light intensity during growth, lower leaves usually have different levels of photosynthesis to upper leaves. Therefore, an erect leaf canopy could, theoretically, increase crop assimilation.

Some evidence supports this hypothesis. For example, some new rice varieties are characterised by erect leaves; this morphological trait allows greater penetration of solar light to the lower leaves, optimising canopy photosynthesis (Duncan *et al.* 1968). In wheat, under fully irrigated conditions, varieties with more erect leaves were associated with up to 11% more biomass and 4% more yield compared with varieties without this trait (Innes and Blackwell 1983). Furthermore, lines with more erect leaf canopies are characteristic of many of CIMMYT's best yielding wheat varieties (Fischer *et al.* 1996). Furthermore, this trait has been associated with higher grain number in barley (Angus *et al.* 1972). Therefore, a significant positive correlation between canopy architecture and yield improvement seems to exist, although there are no available data to determine whether high yielding agronomic types with erect leaf canopy represent a leaf angle "ideotype" or further improvement is possible.

Maintaining the green leaf area, especially after anthesis when there is a rapid decline in leaf area index *(LAI)*, is another way to increase the amount and the duration of light intercepted by the canopy.

Several phenotypes with delayed leaf senescence (i.e. stay-green phenotypes), have been described in crops and, for some of them (maize and sorghum for example), there is a clear evidence for a positive correlation between this trait and increase of yield (Thomas and Smart, 1993). In maize, particularly in the newer hybrids, an increase in biomass is a consequence of delayed leaf senescence and genetic differences in photosynthetic duration have been associated with a longer grain filling duration and higher yield (Moll

et al. 1994, Rajcan and Tollenaar, 1999a). No differences were found between the rates of photosynthesis in these hybrids and in non-stay green types.

Delayed leaf senescence would also increase the photosynthetic output of leaves, especially during the grain filling period of determinate crops. Indeed, it has been calculated that delaying the onset of senescence by just two days will result in increase in carbon fixed by the plant of about 11% (Thomas and Howarth, 2000).

Delayed leaf senescence has become a primary target that can be genetically manipulated by using either conventional or molecular approaches and selection for extended leaf area duration is a worthwhile target in order to increase the duration of crop photosynthesis and hence the yield. A better understanding of the regulation of senescence in photosynthetic tissue is important if this goal is to be achieved.

1.3 The senescence process in plants

1.3.1 General introduction

During its life span, a leaf undergoes at least three phases of development. Initially, it expands rapidly, importing carbon and nitrogen and undergoing rapid protein synthesis until its full capacity for photosynthesis is reached. The mature leaf then becomes an important part of the plant contributing to the supply of carbon. This continues until internal or external conditions initiate the onset of senescence. Metabolically, carbon assimilation (photosynthesis) is replaced by catabolism of chlorophyll and macromolecules.

Leaf senescence is not simply a passive decay mechanism, but rather, it is a highly regulated sequence of events, involving cessation of photosynthesis, disintegration of chloroplasts and loss of chlorophylls (Matile *et al.* 1988, 1989, 1992), breakdown of leaf proteins and removal of aminoacids (Feller and Fischer, 1994) and degradation of total lipids and total fatty acids (Koiwai *et al.* 1981). This is the period of massive mobilisation and recycling of nitrogen, carbon and minerals from the mature leaves to the other parts of the plant. In annual plants, which include most agricultural crops, these nutrients are stored ultimately in the mature seed. Since most of the nitrogen for grain filling is derived from the vegetative organs, remobilisation from senescent leaves is undoubtedly central

for the nutrient budget in seed crops (Feller and Fischer, 1994) and, therefore, leaf senescence may be a limiting factor for the yield in certain crops.

1.3.2. Senescence types

Leopold (1961) has distinguished various senescence types:

1. In annual crop plants with "monocarpic senescence", the death of the vegetative parts is closely linked to fruit maturation (Nooden *et al.* 1997). After reproduction the whole plant dies, as if committing suicide. Monocarpic senescence can be seen in many annual (e.g. soybean, wheat, and barley), some biennials (e.g. carrot) and some perennials (e.g. bamboos) species.
2. "Top senescence" can be observed in plants forming bulbs or tubers (Berchtold *et al.* 1993). In such plants the above–ground parts become senescent and new shoots appear at the beginning of the next season.

3. In deciduous trees, the leaves senesce at the end of the season ("deciduous senescence"), but branches, stems and roots survive (Bortlik *et al.* 1987).

4. "Progressive senescence" can be observed in several groups of perennial plants (e.g. pine) and leaves of such plants may remain active for several years (Feller and Fischer, 1994).

1.3.3. Senescence mechanisms

Although senescence does progress with age, it is not a passive ageing process. Indeed, it is controlled by internal and external signals (**Fig.1.2**), and altering these signals may modify it (Nooden *et al.*, 1997). Environmental stresses such as extreme temperatures, drought, poor light or nutrient supply and pathogen attack will all result in premature initiation of senescence. However, because leaf senescence is an important part of the development of the plant, when a leaf reaches a certain age, senescence will be initiated even if the plant is growing under favourable conditions (Buchanan–Wollaston, 1997).

Several lines of evidence suggest that senescence is an actively regulated process involving co–ordinated expression of specific genes -i.e. programmed leaf senescence- (Smart, 1994; Gan and Amasino, 1997 ; Nam, 1997). However, two other mechanisms have been invoked as causal in the senescence of the vegetative parts. These are 1) nutrient starvation and 2) programmed cell death (PCD).

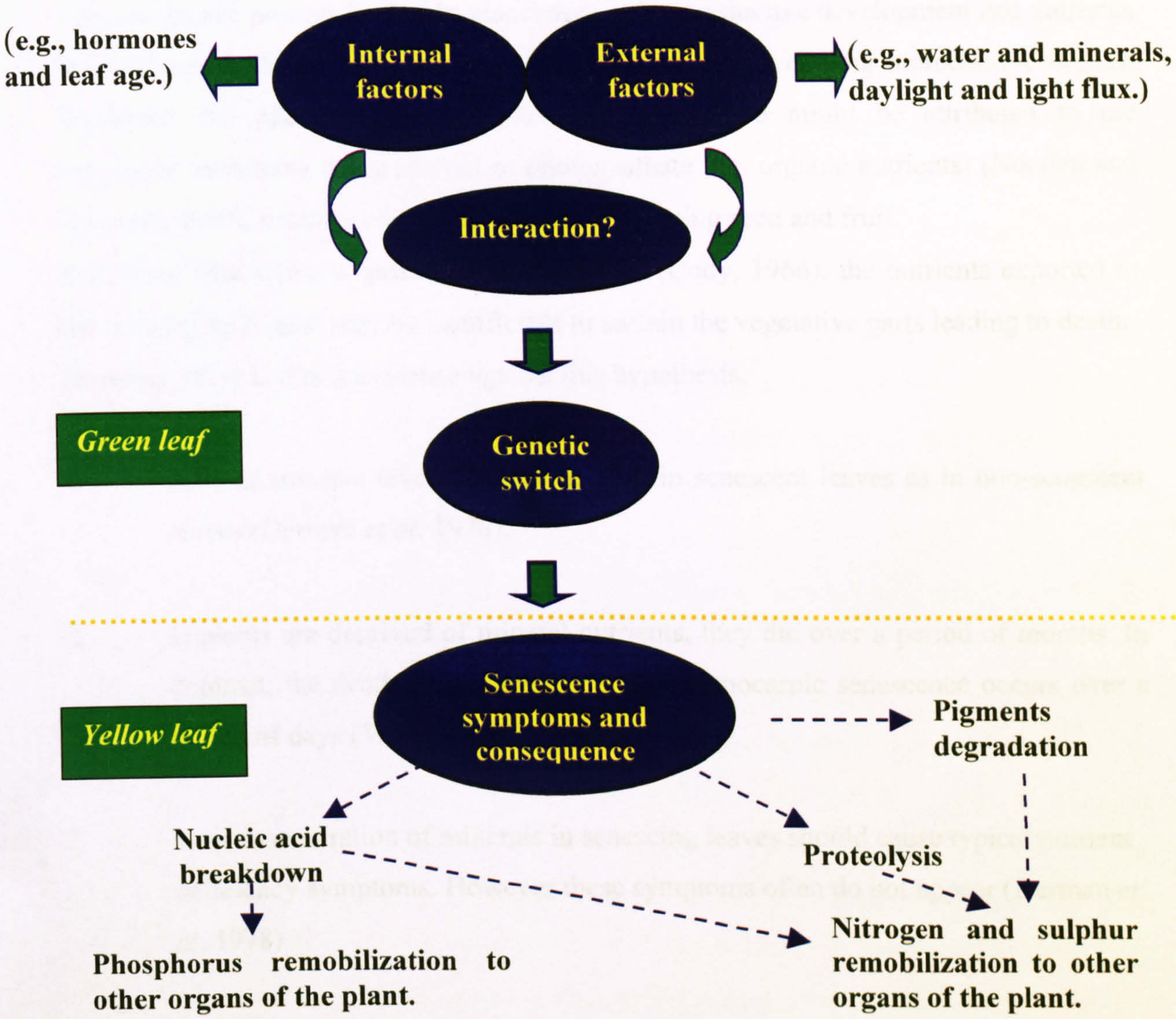


Fig. 1.2. An overview of factors implicated in the initiation of leaf senescence.

By analogy with other biological systems, an initial perturbation ot the system may cause only small changes in control steps resulting in a cascade of secondary effects (adapted from Smart, 1994).

1.3.3.1. *Senescence and nutrient starvation*

The senescence process is closely associated with reproductive development and nutrients are often redistributed from the vegetative parts to the developing reproductive organs. Therefore, the phenomenon of monocarpic senescence might be attributed to the vegetative meristems being starved of photosynthate (i.e. organic nutrients) (Nooden and Guiamét, 1989), because of diversion to the developing seed and fruit.

Following MacArthur's 'principle of allocation' (Cody, 1966), the nutrients exported to the developing organs may be insufficient to sustain the vegetative parts leading to death. However, there is direct evidence against this hypothesis.

1. Mineral nutrient levels may be as high in senescent leaves as in non-senescent leaves (Derman *et al.* 1978).

2. If plants are deprived of mineral nutrients, they die over a period of months. In contrast, the death of vegetative parts in monocarpic senescence occurs over a period of days (Wilson, 1987).

3. Low concentration of minerals in senescing leaves should cause typical nutrient deficiency symptoms. However these symptoms often do not appear (Derman *et al.* 1978).

4. Supplying soybean explants with a mineral nutrient solution does not prevent senescence as the pods develop (Mauk and Nooden, 1992).

This evidence shows that diversion or withdrawal of mineral nutrients does not necessarily cause monocarpic leaf senescence.

1.3.3.2. *Senescence as a form of programmed cell death* (PCD)

Recently, much attention has been focused on leaf senescence as a form of programmed cell death (PCD). It is generally accepted that many plant developmental processes and stress responses are achieved through the operation of PCD. These include senescence, abscission zone formation and the hypersensitive reaction of cells to pathogen infection (Greenberg, 1994; Jones and Dangl, 1996). PCD often, but not always, shows the same specific features, i.e. chromatin condensation, DNA fragmentation (called "laddering", due to its appearance on electrophoretic gel), budding of the nucleus into small bodies and "boiling" of the cytoplasm (Arends, 1996). However, the senescence of organs and leaves appears to differ from typical PCD in several important respects. For example, the cytoplasm does not boil or bud and, perhaps more importantly, the nuclei do not show substantial changes until relatively late in the senescence process (Biradar and Rayburn, 1994). The latter feature is not surprising, because the nucleus must be intact to accomplish the recycling process.

Several lines of evidence suggest that nuclear gene expression is required for leaf senescence. Studies using enucleation (Yoshida and Minamikawa, 1996), selective inhibitors such as actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) (Nooden and Guiamet, 1996), mutants (Thomas, 1987b) and, more recently, genetic engineering (Hensel *et al.*, 1993; Lohman *et al.*; 1994, Gan and Amasino, 1995; Buchanan-Wollaston, 1997), have supported the idea that senescence is an active, genetically programmed process and it is controlled by the nucleus.

However, DNA laddering has been reported in same plants during leaf senescence (Yen and Yang, 1998).

To summarise, leaf senescence is a nuclear–regulated process that allows the mobilisation of nutrients (e.g., nitrogen and sulphur) in the leaves (the source) and their transport and storage in developing organs such as seeds (the sink). However, it is still unclear whether leaf senescence shares any biochemical or genetic pathways with other types of PCD in plants or animals.

1.4. Regulation of leaf senescence

The goal of research in leaf senescence is to understand the regulation of the senescence syndrome. In certain circumstances, external and/or internal factors appear to trigger the process and a decline of photosynthesis often occurs at about the same time as the onset of senescence **(Fig.1.3.a)**. Internal factors such as hormones and reproductive state (such as sink removal and fruit ripening), can either delay or accelerate the onset of the process (**Fig.1.3.b)**. In addition, the leaf senescence programme is accompanied or driven by changes in gene expression and many genes whose transcripts are up or down regulated during leaf senescence have been identified **(Fig.1.3.c)** (Nam, 1997).

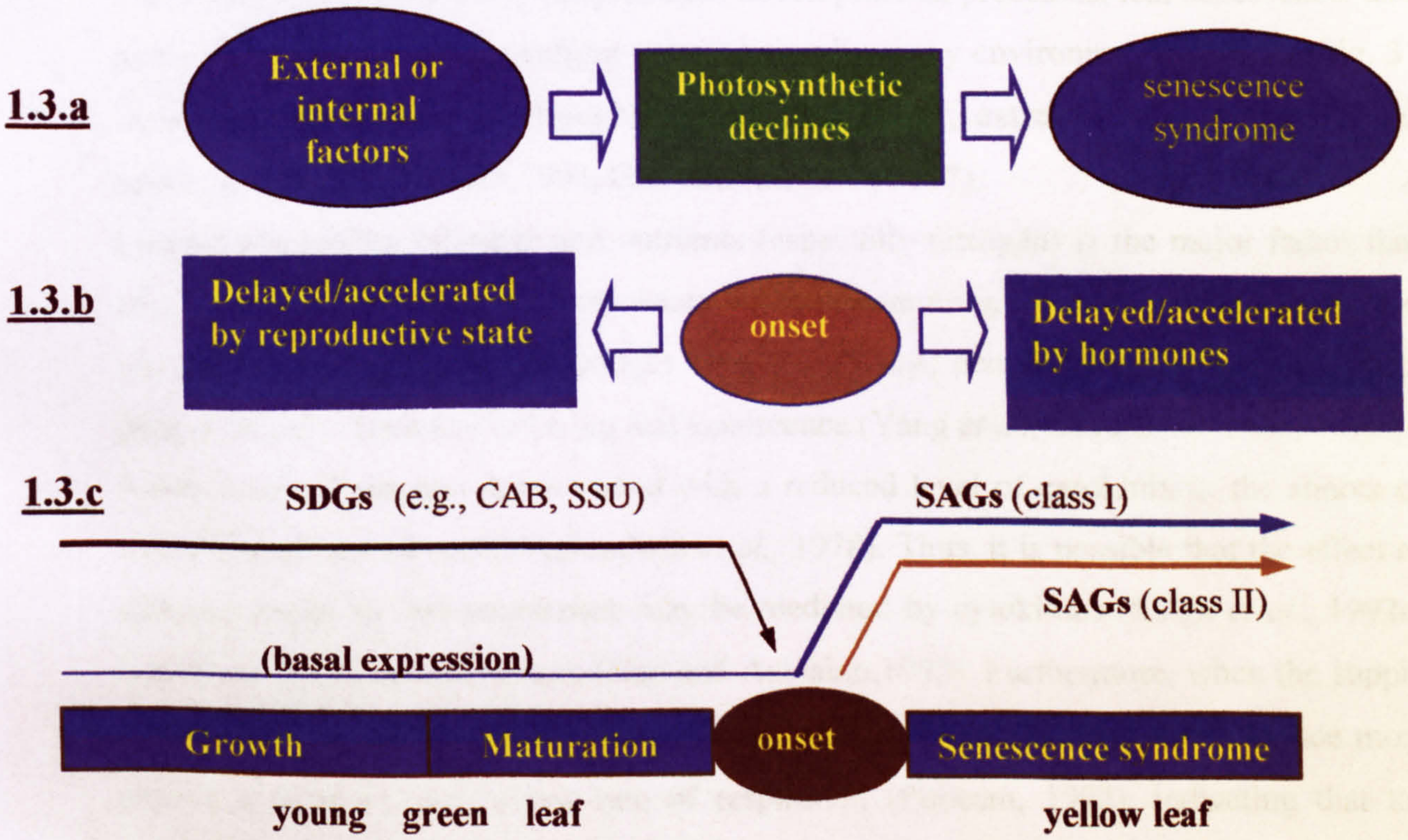


Fig.1.3. A simple model to illustrate the regulation of senescence syndrome.

Some factors such as age and stress lead to a decline in photosynthesis (**Fig.1.3.a**). The initiation of the senescence syndrome can, in certain species or situations, be delayed or accelerated by growth regulators or by reproductive state such as sink removal (**Fig.1.3.b**). The senescence progress is accompanied by or, perhaps, driven by changes in gene expression (**Fig.1.3.c**). Senescence down–regulated genes (**SDGs**) include genes involved in photosynthesis such as the chlorophyll a/b binding protein gene (CAB) and Rubisco small subunit gene (SSU). Senescence associated genes (**SAGs**) are those genes whose expression is up-regulated during leaf senescence. Class I SAGs are expressed only during senescence (senescence-specific). Class II SAGs have a basal level of expression during early leaf development, but this level increases during senescence (adapted from Nam, 1997)

At least four different signalling pathways, can be involved in the regulation of the senescence process:

1. Environmental regulation.
2. Regulation by sugars.
3. Hormonal regulation.
4. Regulation by sink removal.

1.4.1. *Regulation of leaf senescence by the environment*

Like many others genetically-programmed developmental processes, leaf senescence, and particularly its initiation, is subject to regulation by many environmental factors (Fig. 3) including stresses such as drought, nutrient deficiency, extremes of temperature and ozone concentration (Smart 1994, Gan and Amasino, 1997).

Limited availability of water and nutrients (especially nitrogen) is the major factor that adversely affects plant life in many ecosystems. Premature senescence is usually observed when plants are grown under drought stress conditions, because it can negatively affect photosynthetic efficiency inducing leaf senescence (Yang *et al.*, 2000).

A low level of nitrogen is correlated with a reduced level of cytokinin in the shoots or xylem sap of several species (Goodwin *et al.*, 1978). Thus, it is possible that the effect of nitrogen status on leaf senescence may be mediated by cytokinins (Singh *et al.*, 1992a) which are senescence inhibitors (Gan and Amasino,1995). Furthermore, when the supply of nitrogen is low, leaves of *Lolium perenne* with high rate of respiration senesce more rapidly than those with a low rate of respiration (Pilbeam, 1992), indicating that the respiration rate may play a role in foliar senescence. Other mineral deficiencies may either initiate the process (e.g. iron) (Abadia *et al.*, 1991) or not affect it (e.g. phosphorus) (Crafts-Brandner, 1992).

Oxidative damage in a leaf may lead to the initiation of the senescence syndrome (del Rio *et al.*, 1998). In plants such as wheat, small doses of ozone over a period of days may accelerate leaf senescence by production of reactive oxygen species (Gelang *et al.*, 2000).

Low light intensity or darkness results in reduced expression of light-dependent genes and the loss of photosynthetic proteins and chlorophyll (Thomas, 1978). Since phytochrome acts as the light receptor for the expression of many photosynthetic genes, a lower ratio of red:far-red light reaching the lower leaves of a plant can also accelerate the senescence of these leaves (Rousscaux *et al.*, 1996).

Plants have evolved mechanisms by which leaf senescence can be induced by these stresses to reallocate nutrients to reproductive organs and to eliminate water consumption by older, less productive leaves. This regulation of leaf senescence has a clear adaptive value, allowing the plant to complete its life cycle even under stressful conditions (Leopold, 1961).

1.4.2. *Regulation of leaf senescence by sugars*

The most striking event in leaf senescence is the disassembly of the photosynthetic apparatus within the chloroplasts (Thomas, 1982a; Smart, 1994; Matile *et al.,* 1989) and the most clear symptom of this process is the loss of chlorophylls (Matile *et al.*, 1996). Because these phenomena are both associated with a rapid decline of photosynthesis (Jiang, 1993), Blecker and Patterson (1997), have proposed that a decline in photosynthetic activity below a certain threshold level acts as a senescence-inducing signal. It has been known for a long time that photosynthesis is subject to negative feedback regulation in the source–sink relationship (Nooden and Guiamet, 1989) and recent data indicate that elevated sugar levels in the leaf repress the expression of photosynthesis–associated genes (Pego *et al.,* 2000).

If we suppose that cross talk occurs between photosynthesis and the initiation of senescence, it is interesting to identify the signal/(s) that relate photosynthesis to senescence. Because sugars are primary products of photosynthesis, the sugar levels could be one such signal. Indeed, leaves may recognise sugar starvation as a senescence signal and induce the expression of senescence associated genes (SAGs).

Some evidence support this hypothesis:

1. The promoter activities of the *Sen1* (Chung *et al.*, 1997) and *SAG12* (Noh and Amasino, 1999), senescence-associate genes are rapidly induced by sugar deprivation.
2. Exogenously-supplied sugars can repress gene expression in senescent *Arabidopsis* leaves (Noh and Amasino, 1999).
3. When a yeast invertase gene is expressed in the extracellular space of leaf of tomato, *Arabidopsis* and tobacco, carbohydrates are accumulated, photosynthesis is inhibited and the leaves exhibit symptoms that resemble premature senescence (Dickinson *et al.*, 1991, Ding *et al.*, 1993).
4. In non-senescent leaves, sugar accumulation can lead to a decline of chlorophyll and photosynthetic proteins (Krapp *et al.*, 1993).
5. A gene designated *SFP1*, which is similar to monosaccharide transporters, is induced during leaf senescence (Quirino *et al.*, 2001).

However, studies in tobacco plants showed that the levels of glucose and fructose but not of sucrose increased as the leaves progressed through senescence (Wingler *et al.*, 1998). Therefore, variation in sugar content during leaf senescence may occur between species and more studies are required to determine wherever these differences are linked to the senescence process.

1.4.3. *Regulation of leaf senescence by hormones*

Although all the major plant hormones have been proposed to be involved in the senescence process, only cytokinins and ethylene have been conclusively demonstrated to play a role in the regulation of this process **(Fig.1.4).**

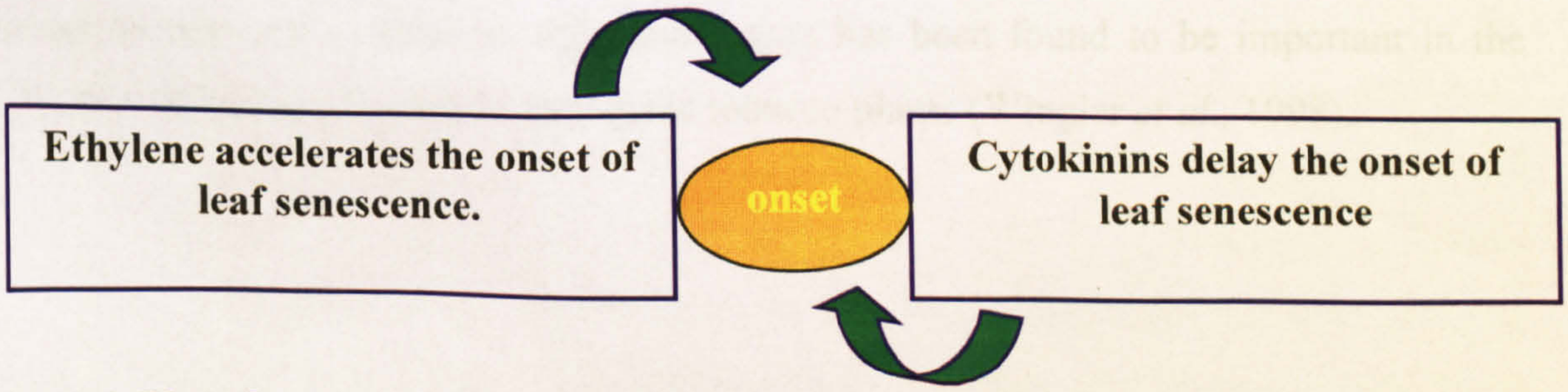


Fig. 1.4. Plant hormones implicated in the senescence process.

Cytokinins and ethylene are two plant hormones which have significant but opposite impacts on leaf senescence.

1.4.3.1. Cytokinins

In many species, functional senescence involves a decline in cytokinin levels in the leaf (Gan and Amasino, 1995). Cytokinins are synthesised mainly in the root and transported to the rest of the plant in the xylem. It has been shown that the level of cytokinins in the xylem sap declines when senescence is initiated and it has been suggested that a reduced level of cytokinins in the leaf may cause the onset of senescence (Nooden *et al.*, 1988b). Furthermore, external application of cytokinin to a detached leaf or a localized leaf area results in the retention of greenness (Richmond and Lang, 1957).

Recently, the ability to transform plants with foreign genes has made it possible to alter the endogenous cytokinin level. Transgenic plants with altered levels of cytokinins due to the expression of a bacterial gene encoding isopentenyl-transferase (IPT, the enzyme that catalyses the limiting step in cytokinin biosynthesis) have been produced (Smart *et al.*,1991, Gan and Amasino, 1995). The promoter of a senescence-specific *Arabidopsis* gene, encoding a protease (Lohmam, 1994), has been used to direct IPT expression in tobacco plants **(Fig.1.5)** (Gan and Amasino, 1995). The transformed plants developed similarly to the control plants and only the senescence process was significantly retarded in leaves from the transformed plants. As a consequence of the delay in senescence, the photosynthetic activity in the leaves of the transgenic plants remained higher than in untransformed plants, more flowers were produced and an increased seed yield was observed (Gan and Amasino, 1995). The control of senescence by cytokinins is likely to be at the transcriptional level as the presence of cytokinins above a certain level inhibits the expression of senescence-related genes (Buchanan-Wollaston, 1997). Recently, an interaction between cytokinins, light and sugars has been found to be important in the regulation of leaf senescence in transgenic tobacco plants (Wingler *et al.*, 1998).

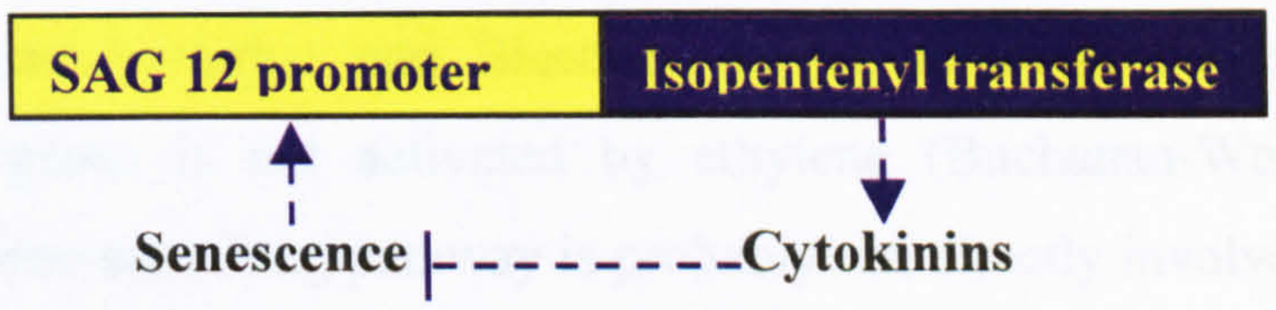


Fig.1.5. Retardation of leaf senescence by autoregulated production of cytokinins.

The senescence-specific SAG 12 promoter was fused to a cytokinin-synthesising gene, isopentenyl transferase. The onset of senescence activates this promoter to direct the production of cytokinins. Cytokinins in turn inhibit senescence, thus attenuating the SAG promoter activity to prevent overproduction of cytokinins (adapted from Gan and Amasino, 1995).

1.4.3.2. Ethylene

In contrast to cytokinins, ethylene treatment often promotes senescence as it accelerates many of the physiological changes normally associated with leaf senescence (Aharoni *et al.,* 1979; Gepstein and Thimann, 1981). However, although ethylene plays a regulatory role in many plant processes (Theologis, 1993, Borochov and Woodson, 1989), in some plants (e.g., strawberry and monocots) it appears to play no role during processes such as fruit ripening and senescence (Smart, 1994; Valpuesta *et al.,* 1995). In addition, recent evidence from transgenic plants and ethylene responsive mutants have indicated that, although ethylene has an effect on senescence, it is not an essential regulator of the process. For example, mutants of tomato that are defective in the ethylene signal transduction pathway have been used to study the effects of ethylene during both fruit ripening and senescence. Plants of tomato carrying the *never ripe* mutation, which has been shown to block ethylene perception, produce fruit that ripen extremely slowly (Lanahan *et al.,* 1994), but the leaves show only a small delay in the onset of senescence. Transgenic tomato plants in which ethylene synthesis was blocked by the introduction of an antisense 1-amino cyclopropane 1-carboxylic acid (ACC) oxidase gene, showed delayed leaf senescence (John *et al.,* 1997). However, once senescence had been initiated, the expression level of SAGs did not differ greatly from that of wild-type plants. These effects are comparable with results obtained with the ethylene-insensitive *Arabidopsis* mutant, *etr-1* (Grbic and Bleecker, 1993).

The role of ethylene in leaf senescence appears to be as a modulator in determining the time of leaf senescence (Grbic and Bleecker, 1993), although the transcription of senescence-related genes is not activated by ethylene (Buchanan-Wollaston, 1997). Therefore, the ethylene-signalling pathway is probably not directly involved in regulating the transcription of genes during leaf senescence although the mechanism/(s) by which ethylene modulates the rate of senescence is still unclear.

Others hormones such as abscisic acid, jasmonates, auxins, gibberellins (Smart, 1994) and, recently, brassinosteroids (Clouse, 1996), can either accelerate (e.g., abscisic acid and jasmonates) or delay the senescence process (e.g., auxins, gibberellins and brassinosteroids). However, it is not yet clear if they are directly involved in the process or are only secondary effects.

1.4.4. *Regulation of leaf senescence by sink removal*

The reproductive structures of most crop species are the ultimate sink for a large portion of minerals and assimilates transported in the plant and they can also influence many physiological processes, including leaf photosynthesis and leaf or whole plant senescence (Nooden and Leopold, 1988). Although some effects of reproductive sinks can be attributed to the removal or alteration of translocated nutrients and/or to plant growth regulators (Biswas and Mandal, 1986), it has been postulated that the sinks may also be sources of regulatory substances responsible for these effects-i.e. "the death hormone hypothesis"-(Engvild, 1989). "The death hormone" is proposed to be a senescence factor produced by seeds, fruits or flowers and to be transported (in the xylem) to the vegetative organs **(Fig.1.6)**. It induces cessation of growth and remobilization of nutrients to the sinks (Kelly and Davies, 1988). However, although the existence of this factor has certainly not yet been proven (Gianfagna and Davies, 1997), some evidence suggests a cross talk between sink and source in the regulation of leaf senescence.

1. In soybean, leaf senescence is retarded or even inhibited if the plant is prevented from flowering or setting fruit (Nooden and Murray, 1982).
2. In wheat and rice removal of the ear delayed leaf senescence (Patterson and Brun, 1979; Biswas and Choudhuri, 1981).

3. In peas (*Pisum sativum* L.) removal of the fruit prevents apical senescence (Hamilton and Davies, 1988).

4. Tomato plants that overproduce ethylene produce no flowers or fruits and leaf senescence does not occur, possibly because of lack of signals from the developing fruits (Lanahan *et al.*, 1994).

Ironically, *Arabidopsis thaliana,* which has become a model for studying developmental processes, does not show control of monocarpic senescence by the reproductive structures (Hensel *et al.,* 1993). Indeed, single-gene mutations in *Arabidopsis* that cause male sterility (e.g., *male-sterile 1*), delayed flowering (e.g., *constans*), or early termination of the inflorescence (e.g., *terminal flower 1*) have no effect on timing of leaf senescence (Hensel *et al.,* 1993).

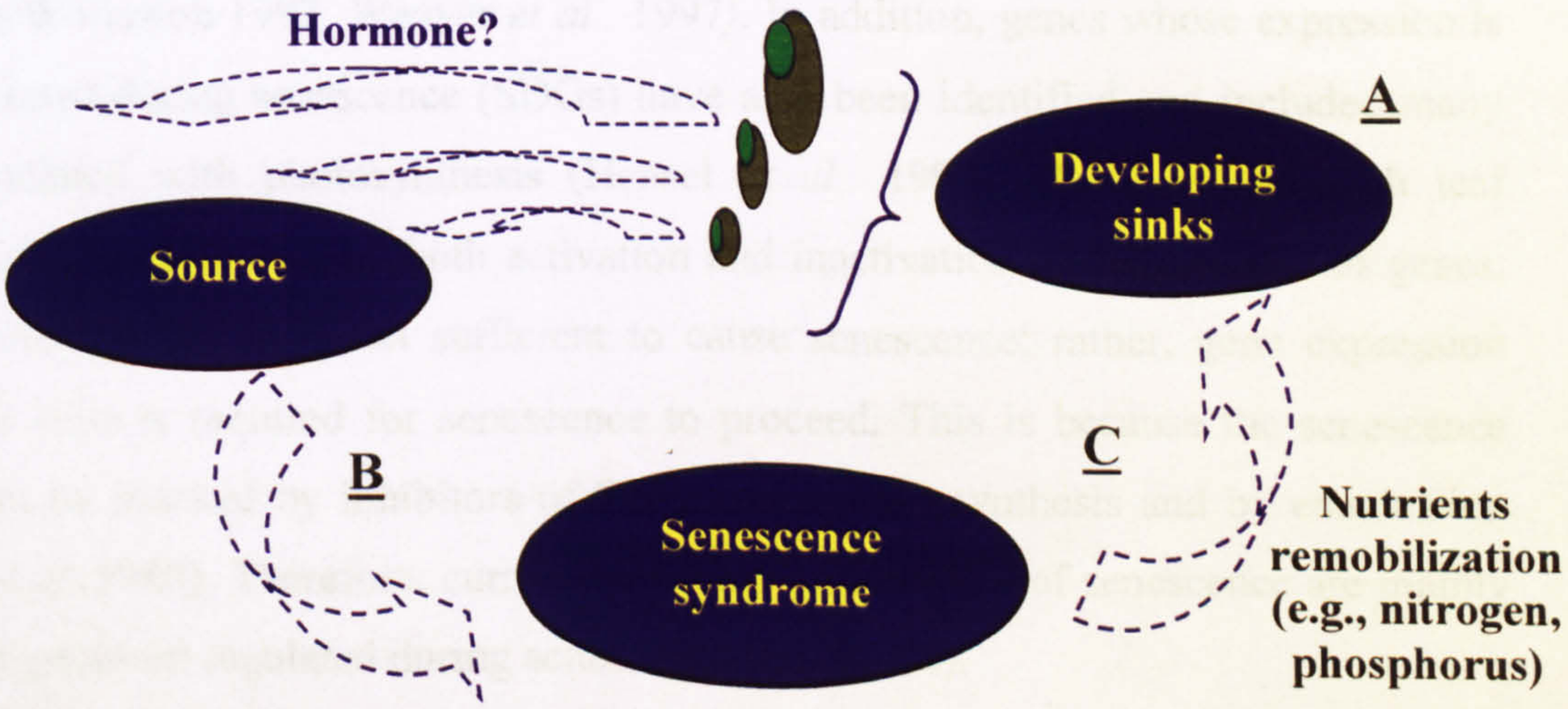


Fig.1.6. "The death hormone hypothesis".

A factor (hormone?) produced by the sinks (**A**) (e.g., seeds, fruits or flowers) is transported to the source where it induces leaf senescence (**B**) and remobilization of nutrients (**C**).

1.5. Genes expressed during leaf senescence

Despite gaps in our understanding of the process, it is clear that senescence syndrome is a genetically controlled process that requires continuing gene expression. An important aim of molecular studies of leaf senescence is to identify and clone genes encoding enzymes

that are involved in the senescence process, to characterise these genes and to determine their function in order to obtain an overall view of the enzymatic changes that take place. Furthermore, once a senescence gene has been identified, the mode of regulation of this gene can be studied. Analysis of the mechanism of regulation of senescence genes will provide important clues to the molecular mechanism of initiation and progression of leaf senescence.

Using both differential screening and substractive hybridization techniques, a number of cDNA clones encoding either senescence-down regulated genes (i.e. SDGs) or senescence-up regulated genes (i.e. SAGs) have been identified from a range of different plants (Hensel *et al.*,1993, Smart *et al.*,1995, Buchanan-Wollaston 1997*)*. The deduced amino acid sequences of at least some SAGs indicate possible roles in salvage of lipids, proteins and nucleic acids. Indeed, cDNAs with sequence similarities to proteases, nucleases and proteins involved in nitrogen and lipid metabolism have been identified (Buchanan-Wollaston 1997, Weaver *et al.*, 1997*)*. In addition, genes whose expression is down-regulated during senescence (SDGs) have also been identified and included many genes associated with photosynthesis (Hensel *et al.*, 1993). However, although leaf senescence is characterised by both activation and inactivation of distinct sets of genes, gene inactivation *per se* is not sufficient to cause senescence; rather, gene expression within leaf cells is required for senescence to proceed. This is because the senescence process can be blocked by inhibitors of RNA and protein synthesis and by enucleation (Nooden *et al.*,1988). Therefore, current molecular studies on leaf senescence are mainly focused on genes up regulated during senescence (i.e. SAGs).

Physiological parameters such as chlorophyll content and photosynthetic rate have been used to determine the timing of leaf senescence and to identify the different stages of senescence at which the expression of a specific gene is induced (Smart *et al.*,1994). Furthermore, on the basis of the patterns of expression and the functions of the protein products, several classes of genes that could be involved in leaf senescence as SAGs were proposed **(Fig.1.7)** (Buchanan-Wollaston, 1997).

A. Class A includes regulatory genes that are expressed at the initiation of senescence and control its timing and rate of progress. Genes of this type may be cloned infrequently since they are likely to be expressed at low levels. To date,

only few genes of this class have been identified (Wilkinson *et al.*,1995; Schaller and Bleecker, 1995; Hajouj *et al.*, 2000).

B. Class **B** includes genes with increased levels of expression at the SS1 stage, when chlorophyll levels are just starting to decrease. Genes encoding for cysteine proteinase (Lohman *et al.* 1994; Smart *et al.*, 1995), ACC oxidase (Davies and Grierson, 1989) and glutathione S-transferase (Smart *et al.*, 1995) all belong to this class.

C. Class **C** consists of genes involved in the mobilisation of storage products, although they may also function during other developmental stages [e.g., malate synthase and isocitrate lyase (Graham *et al.* 1992, Mc Laughlin and Smith, 1994)].

D/E. Class **D** and **E** genes are similar in terms of period of expression, but their mRNAs increase differently at a particular stage of senescence. In particular, class E genes maintain their basal level of expression just before the beginning of the senescence process and then it increases rapidly. Genes encoding for glutamine synthetase (class **D**, Buchanan-Wollaston and Ainsworth, 1997), ferritin (class **D**, Buchanan-Wollaston and Ainsworth, 1997), catalase (class **D**, Buchanan-Wollaston and Ainsworth, 1997) and PR 1a (class **E**, Hanfrey *et al.* 1996) are examples of these two classes.

F. Class **F** includes genes such as asparagine synthetase *(*King *et al.* 1995) and ATP sulphurylase (Buchanan-Wollaston and Ainsworth 1997) whose expression is induced at specific stage during senescence; however, in contrast to class **B** genes, expression does not continue to the final stages of senescence.

G. The final class of genes, termed class **G**, is strongly expressed in leaf development [e.g., pyruvate *o*-phosphate dikinase, (Smart *et al.*1995)] and again during leaf senescence.

In summary, a large number of cDNA clones representing genes that show induced or enhanced levels of expression during leaf senescence have been identified. Although the loss of chlorophyll is readily observed during senescence (due to its breakdown during chloroplast disassembly), many other catabolic events such as protein, lipid and nucleic acid degradation occur. Therefore, it is not surprising that the majority of SAGs identified so far are involved in the recycling of these components.

1.6. Regulation of senescence-associated gene

Many leaf SAGs are not uniquely induced during senescence. Analyses of the expression of SAGs in response to different senescence-inducing treatments have been used to address the extent of overlap between age-dependent leaf senescence and senescence induced by other factors. Studies with ABA, ethylene, cytokinin, dehydration, dark treatment (Gan and Amasino, 1997; Park *et al.,* 1998), heat shock (Gensghik *et al.,* 1995) and nutrient starvation (Bariola *et al.,*1994) have shown that these genes are differently regulated, suggesting that there are multiple signalling pathways leading to their induction; certain genes are likely to share common pathways, while others may have unique, specific pathways **(Fig.1.8)**. The range of different expression patterns identified by northern analysis (Fig.1.7) and the changes in expression patterns in response to different treatments or conditions (Fig.1.8), imply that the transcription of these senescence-related genes is not regulated by the same mechanism. Indeed, if a single regulatory transcriptional factor was involved in senescence-associated gene expression, SAGs should have a common *cis*-acting element in their promoter regions. Comparison of the upstream sequences of several SAGs did not reveal any regions of similarity (Gan and Amasino 1995; Oh *et al.,* 1996); therefore the regulatory elements conferring senescence expression remain to be identified.

However, the SAG12 promoter (and its orthologues in *Brassica napus* BnSAG12-1 and BnSAG12-2) confers senescence-specific expression in *Arabidopsis*, tobacco and *Brassica napus* suggesting that the regulatory elements are conserved across species **(Fig.1.9)** (Sun and Amasino,1999).

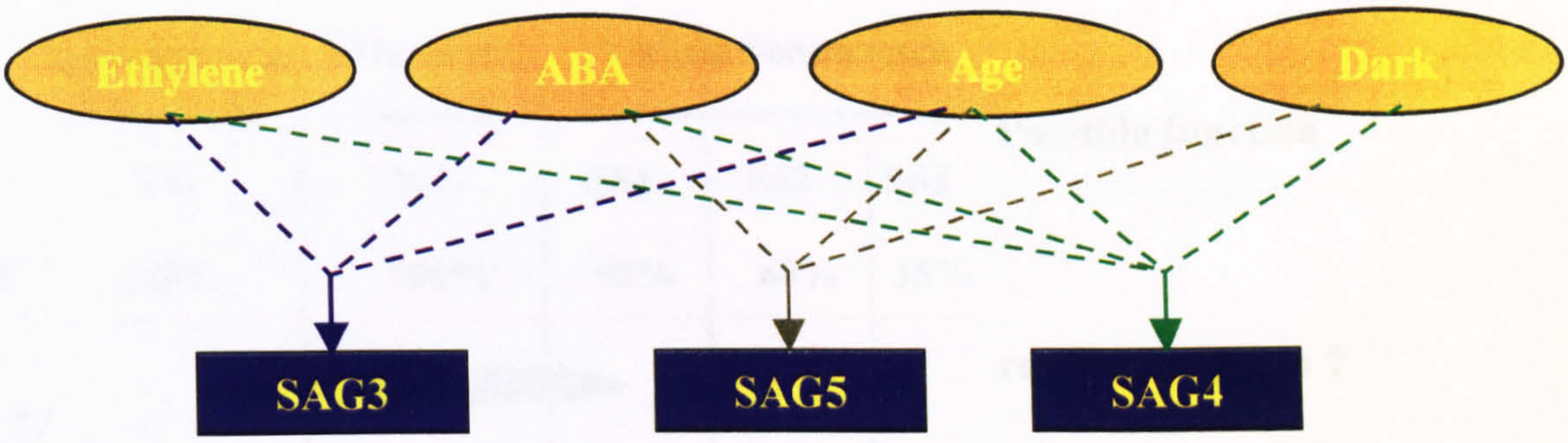


Fig.1.8. The involvement of different sets of genes during leaf senescence.

Although the symptoms of senescence may look the same, the molecular states of senescent leaves are different depending on the senescence factors.

For example, the SAG5 gene may be involved in ABA, age and dark-induced senescence but not in ethylene induced-senescence. In contrast, the SAG4 gene is involved in senescence affected by all the factors (adapted from Nam, 1997).

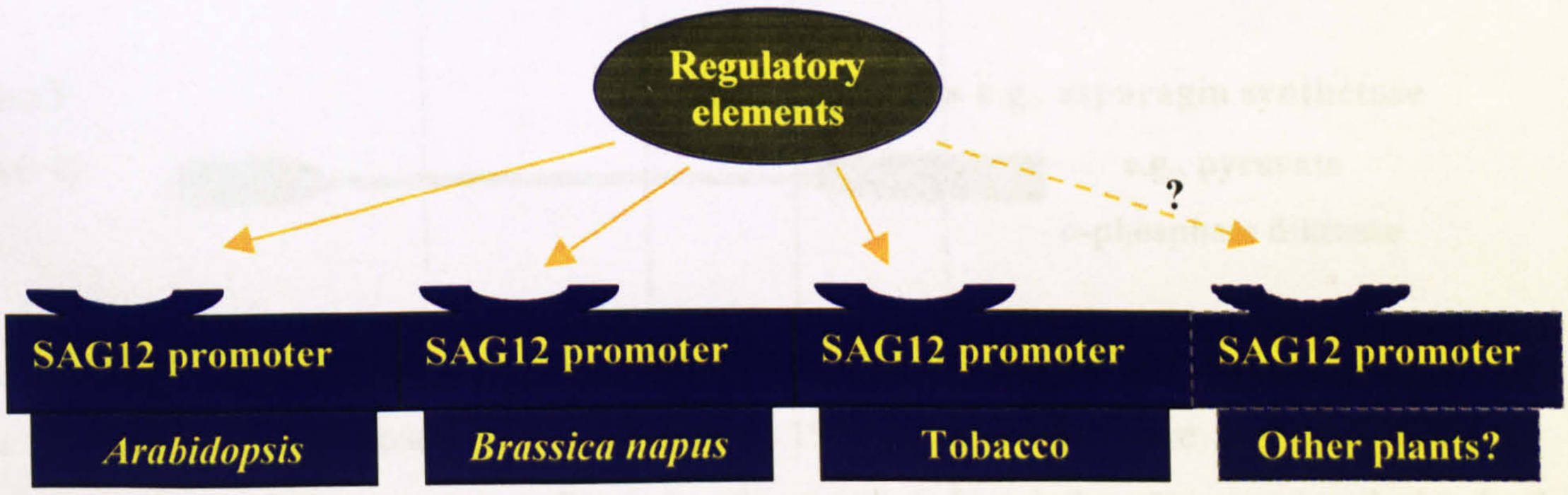


Fig.1.9. SAG12 promoter regions are conserved between species.

The SAG12 promoter can drive the senescence-specific expression of either isopentenyl transferase or GUS in tobacco (Gan and Amasino,1995). The expression patterns of its othologues in *Brassica napus* (BnSAG12-1 and BnSAG12-2), have demonstrated that the senescence-specific regulation of this class of cysteine proteases is conserved across these species. This suggests that the DNA-binding proteins should also be conserved across these three species. Other plants distantly related to *Arabidopsis* might share the same mechanism.

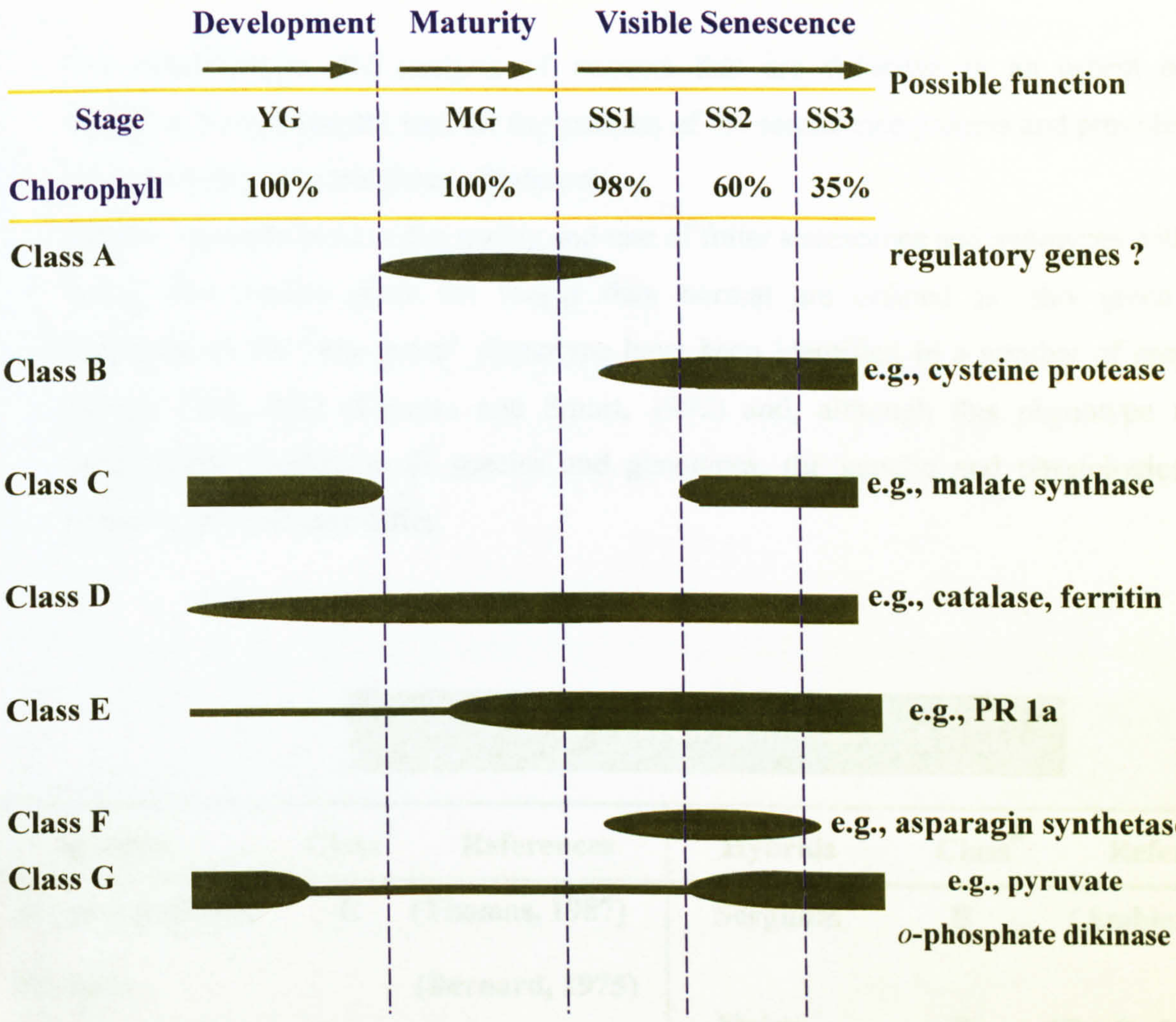


Fig.1.7. Expression of senescence associated genes (SAGs) during leaf senescence.

Classes A-C are as proposed by Smart (Smart, 1994). Classes D-G are proposed based on analyses of the expression patterns of a range of genes that show induced expression during leaf senescence in *Brassica napus* (Buchanan-Wollaston, 1997). The stages of development are those for RNA isolation and gene expression analysis. **YG** refers to RNA isolated from fully expanded green leaves from young plants. **MG** refers to RNA isolated from mature green leaf from plants that have just started flowering. **SS1**, **SS2** and **SS3** refer to RNA isolated from senescent leaves showing 98%, 60% and 35% of green leaf chlorophyll levels, respectively. The possible functions of the gene products have been deduced by comparison with the sequences of previously characterised genes published in DNA and protein databases (adapted from Smart, 1994 and Buchanan-Wollaston ,1997).

1.7. Genetic analysis of leaf senescence

The identification and analysis of mutants that are defective in an aspect of senescence is a powerful tool for the analysis of the senescence process and provides an opportunity to clone genes of interest.

Genetic variation exist in the timing and rate of foliar senescence and genotypes with leaves that remain green for longer than normal are defined as 'stay green'. Examples of the 'stay green' phenotype have been identified in a number of crop species (**Tab. 1.1**) (Thomas and Smart, 1993) and, although this phenotype is superficially similar in all species and genotypes, the genetic and physiological routes to the trait may differ.

Stay green genotypes

Mutants	Class[a]	References	Hybrids	Class[a]	References
Festuca pratensis	C	(Thomas, 1987)	Sorghum	B	(Ambler *et al.* 1987)
Soybean		(Bernard, 1975)	Maize	B	(Crafts-Bradner *et al.*, 1984)
Pisum sativum	C	(Thomas *et al.*, 1996)			
Tomato	C	(Akhtar *et al.*, 1999)			
Arabidopsis		(Oh *et al.*,1997)			

Table 1.1. Different types of 'stay green' genotypes in plants.

Examples of the 'stay green' phenotype have been identified in a number of crop species either as mutants or as hybrids.

[a] The possible class is based on the 'stay green' behaviour.

At least five types of 'stay green' behaviour can be distinguished in plants (**Fig.1.10**). Classifying stay-green mutants in this way is useful for understanding the types of modified genes or physiological processes underlying the phenotype but, in practice, a particular 'stay green' phenotype can result from a combination of one or more different functional types (Thomas and Howarth, 2000).

Type **A** and **B** 'stay green' mutants may result from alteration of genes involved, respectively, in the timing of the initiation of senescence and the regulation of its rate of progress. Since this 'stay green' phenotype continues to photosynthesise for longer than normal, it might be expect to show a higher yield in crops (i.e. is functionally 'stay green').

In contrast, types **C** and **D** look green but lack photosynthetic competence either due to premature death, such as that caused by harvesting for food (type D), or by lesions in chlorophyll catabolism.

In type **E** behaviour, the photosynthetic capacity may follow the normal ontogenetic pattern, but comparison of absolute pigment contents identifies it as 'stay green'.

Genes involved in the generation of type A 'stay-green' phenotype are likely to belong to the class A group of senescence associated genes (Fig.1.7), while the genes affected in types B and C are more likely to be from class B (Fig.1.7). However, the gene(s) responsible for these phenotypes have not yet been cloned.

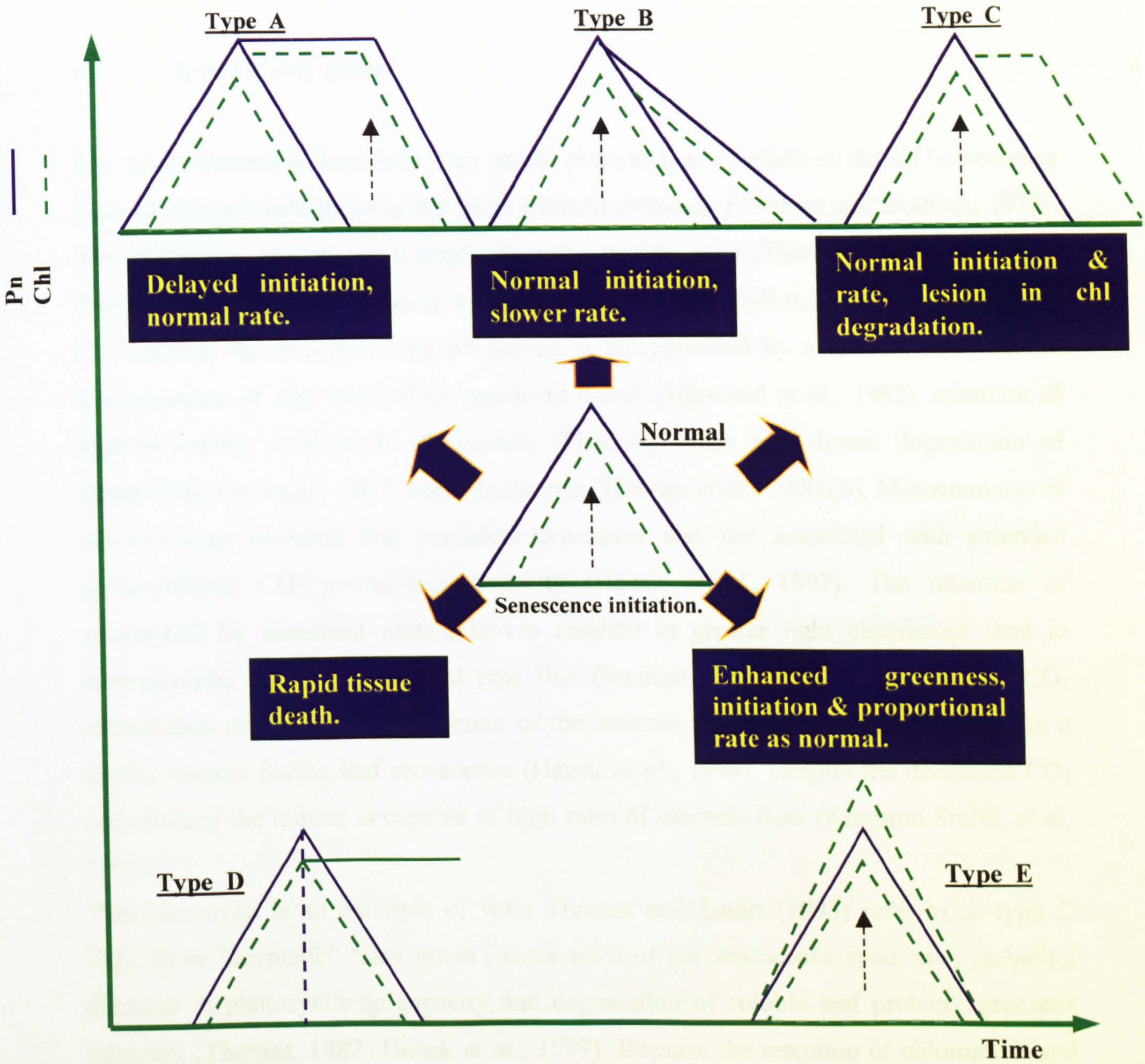


Fig.1.10. Characteristics of different forms of 'stay-green' behaviour in plants.

Graphs show chlorophyll contents (- - -) and photosynthetic capacity (——) (arbitrary scale) for a representative leaf, whole plant or canopy. Initiation of the entire senescence syndrome may be delayed (**Type A**). The syndrome may start on time, but proceeds at a reduced rate (**Type B**), or one or more of the constituent metabolic processes may be disabled (**Type C**). **Type D** represents the case of plant materials that retain greenness because they are rapidly killed at harvest. In **Type E** photosynthetic capacity may follow the normal pattern but absolute pigment content identifies it as 'stay-green' (adapted from Thomas and Howarth, 2000).

1.8. Type C 'stay green'

The most extensively described 'stay green' mutants is the *y* allele of the *sid* (*senescence induced degradation*) locus in the grass *Festuca pratensis* (Thomas and Stoddart, 1975). The mutation is inherited as a single recessive nuclear gene (Thomas, 1987b) and green tissue of the individuals homozygous for *sid*^{y} retain chlorophyll more or less indefinitely. Furthermore, the non-yellowing phenotype is characterised by a marked delay in the disappearance of acyl lipids from senescent leaves (Harwood *et al.*, 1982), retention of light-harvesting chlorophyll a/b protein (Thomas, 1982) and slower degradation of carotenoids (Gut *et al.*, 1987) and cytochrome f (Davies *et al.*, 1990a,b). Measurements of gas exchange revealed that persistent greenness was not associated with extended photosynthetic CO_2 assimilation capacity (Hauck *et al.*, 1997). The retention of chlorophyll by senescent mutant leaves resulted in greater light absorbance than in corresponding leaves of the wild type line (Souriau *et al.*, 1995), however the CO_2 assimilation rate and Rubisco content of the mutants and wild type plants decline in a similar manner during leaf senescence (Hauck *et al.*, 1997). Despite the decreased CO_2 assimilation, the mutant is capable of high rates of electron flow (Kingston-Smith, *et al*, 1997).

This phenotype is an example of what Thomas and Smart (1993) refer to as type C (Fig.10) or "cosmetic" 'stay green', since most of the senescence syndrome, including decrease in photosynthetic capacity and degradation of soluble leaf proteins, proceeds normally (Thomas, 1982; Hauck *et al.*, 1997). Because the retention of chlorophyll and thylakoid proteins represent about 25% of the total nitrogen in a mature leaf (Evans, 1988), a significant proportion of leaf protein N is immobilised in the old, non-photosynthetic leaves of this class of 'stay green' mutant. Indeed, higher levels of total nitrogen in senescent leaves of *Festuca* mutant than in wild type plants were found (Hauck *et al.*, 1997). Furthermore, it was shown in *Lolium perenne* that the 'stay green' trait had significant consequences for plant nitrogen relations, with higher 'sink strength' of shoots for recently absorbed N compared with those of normal plants. However, although the N-use efficiency might be expected to be lower in this class of 'stay green'

mutants than in normal plants, there were no differences in rates of dry matter production (Bakken *et al.*, 1997).

1.8.1. Biochemistry and genetics of sid^y locus

Current knowledge of the pathway of chlorophyll catabolism suggests that the pigment is converted to colourless catabolites in essentially three steps catalysed by 1) chlorophyllase, 2) Mg-dechelatase and 3) phaeophorbide (phaeide) *a* oxygenase (**Fig.1.11**). Theoretically, the loss of catalytic function in any one of these enzymes would cause a "stay green" phenotype (Vincentini *et al.*,1995). The nature of the biochemical lesion in the type C 'stay green' genotypes has been identified as a deficiency in the chlorophyll-degrading enzymes (Bachmann *et al.*, 1994; Vincentini *et al.*, 1995; Thomas *et al.*, 1996). For example, the 'stay green' mutant of *Festuca pratensis* is competent with regard to all known components of the chlorophyll catabolic pathway, except for phaeide *a* oxygenase residing in the thylakoids of senescent leaves (see Fig.11 which is adapted from Vincentini *et al.*, 1995).

Festuca pratensis is perennial and is a genetically heterogeneous outbreeding plant. However, the mutant gene (sid^y) has been introgressed into a range of *Lolium* backgrounds, via intergeneric hybrids with *Lolium multiflorum* L. and *Lolium perenne* L., to give a fast-growing, annual inbreeding grass called *Festulolium* (Thomas *et al.*,1997). Crosses were also made between diploid phenotypically 'stay green' individuals from a segregating *Festulolium* population and the inbreeding annual species *Lolium temulentum*. Senescing leaves of *L. temulentum* plants homozygous for the mutant allele of *sid* (*yy*) retained greenness indefinitely. Furthermore, heterozygotes plants (*Yy*) were phenotypically identical to the wild type plants, confirming that *y* allele was fully recessive to *Y* allele (Thomas *et al.*, 1999).

Although a similar phenotype has been observed in other plants (Thomas *et al.*, 1999) and none of the corresponding genes has yet been cloned, the introgression of the mutant gene (sid^y) into new backgrounds (with production of near-isogenic lines), has allowed more detailed studies of its location and expression.

Indeed, the gene has been mapped on chromosome 6 of *Festuliolium*, two associated AFLP markers have been identified and a small number of unknown genes that could be related to the mutation have been identified (Thomas *et al*., 1997).

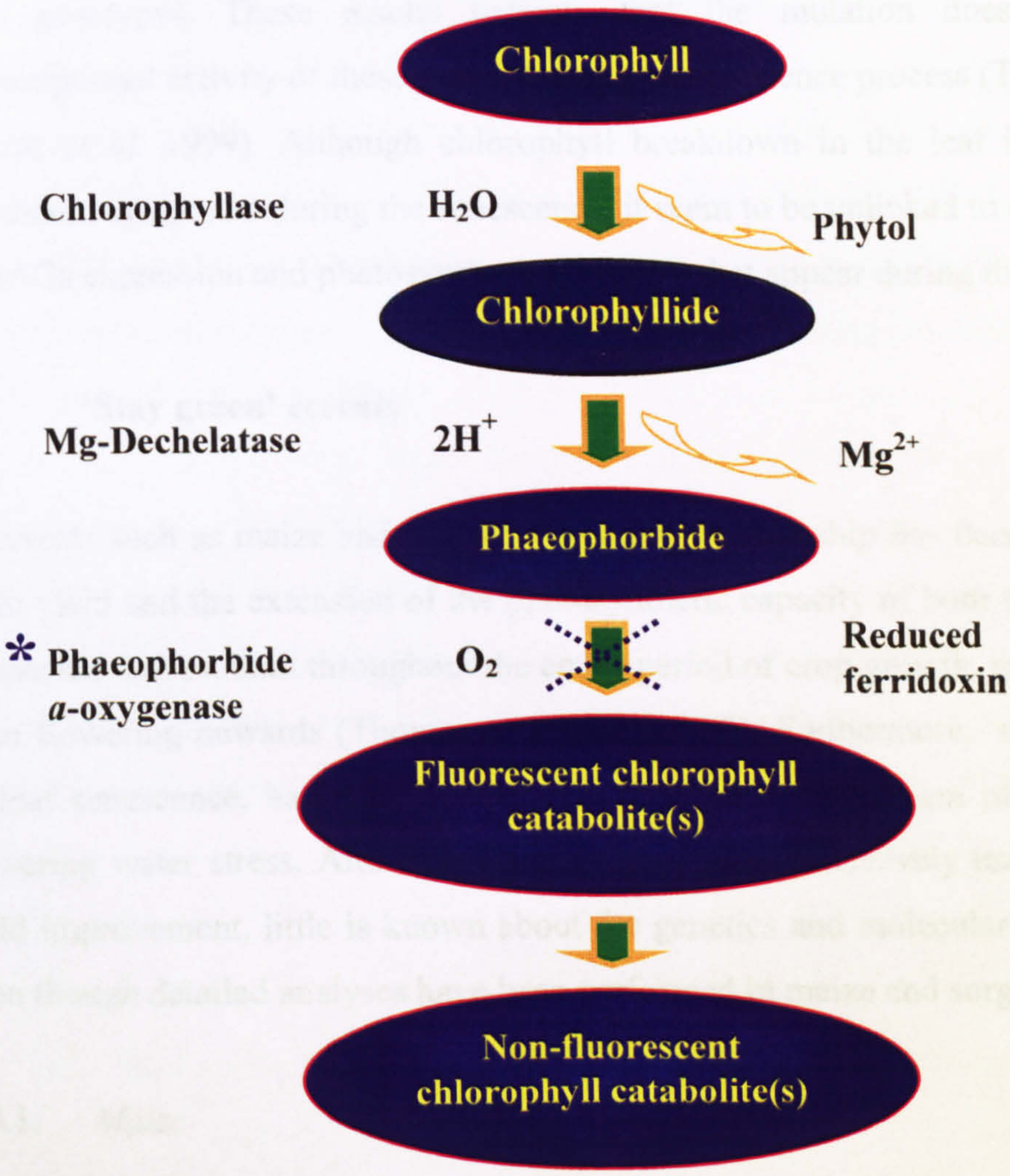


Fig.1.11. The pathway of chlorophyll degradation in senescent leaves.

Current knowledge of the pathway of chlorophyll catabolism suggests that the pigment is converted to colourless catabolites in essentially three steps, catalysed by **1**) chlorophyllase, **2**) Mg-dechelatase and **3**) phaeophorbide (phaeide) *a* dioxygenase.

*The "stay green" mutant of *Festuca pratensis* (and other type C 'stay green' mutants) is competent with regard to all known components of the chlorophyll catabolic pathway, except for phaeide *a* dioxygenase residing in the thylakoids of senescent leaves (adapted from Vincentini *et al*., 1995).

1.8.2. Type C 'stay green' mutants and SAGs expression

Analysis of the expression of senescence-related genes which are either up or down regulated in type C 'stay-green' mutants, reveals identical trends for both mutant and wild type genotypes. These results indicate that the mutation does not modify the transcriptional activity of these genes during the senescence process (Thomas *et al.*, 1992; Akhtar *et al.* 1999). Although chlorophyll breakdown in the leaf is one of the most prominent symptoms during the senescence, it seem to be unlinked to other features (such as SAGs expression and photosynthetic capacity) that appear during the same process.

1.9. 'Stay green' cereals

In cereals such as maize and sorghum, a strong relationship has been observed between grain yield and the extension of the photosynthetic capacity of both the total canopy and of specific leaves, both throughout the entire period of crop growth, and during the period from flowering onwards (Thomas and Smart, 1993). Furthermore, 'stay green', or delay of leaf senescence, has usually been described as a mechanism of resistance to post-flowering water stress. Although breeders have been extensively used such material for yield improvement, little is known about the genetics and molecular biology of this trait even though detailed analyses have been performed in maize and sorghum.

1.9.1. *Maize*

Improvements in grain yield and some other agronomic traits have been linked to increased late season plant health, or 'stay green' in maize (Rosenow and Clark; 1981; Russell, 1985). In maize, non-'stay green' genotypes start losing the green colour of their leaves approximately 30 days after anthesis, while 'stay green' genotypes do not show a similar effect until physiological maturity (Crafts-Brandner *et al.*, 1984). In two synthetic maize populations, analysed for correlations between 'stay green', stalk water content, leaf water content, vegetative period and grain moisture (Bekavac *et al.*, 1998), either the leaf water content or the stalk water content was found to be the most important trait in determing the 'stay green' phenotype. Furthermore, a study of 10 short-season maize

hybrids showed a positive correlation between leaf area duration and yield (Tollenaar, 1978), and some inbred lines that exhibit delayed senescence were characterised by higher water and chlorophyll contents in the leaves at maturity and high sucrose content during grain filling (Gentinetta *et al.*, 1986).

One 'stay green' variety which has been studied in some detail is FS854, which is credited with the world record yield for non-irrigated maize. After ear removal, FS854 remained green and showed an increase in leaf dry weight and sugar content throughtout the grain filling period (Crafts-Brandner *et al.*, 1984). In intact FS854 plants, the levels of chlorophyll and PEP carboxylase start to decline as in wild type lines, but the rate of decrease is reduced compared with other varieties, suggesting that FS854 may be a type B 'stay green' (Thomas and Smart, 1993).

In agronomic terms, 'stay green' maize varieties, with longer leaf area duration than normal, have the advantage of a larger kernel weight. Therefore, the benefits of increased yield by 'stay green' varieties have been exploited successfully by maize breeders (Duvick, 1984). Indeed, new hybrids of maize, may have an increase in biomass resulting delayed leaf senescence, and genetic differences in photosynthetic duration have been associated with a longer grain filling duration and higher yield (Moll *et al.*, 1994).

1.9.2. *Sorghum*

The term 'stay green' has been used to describe the post-flowering drought tolerance response in sorghum (Rosenow and Clark, 1981). This is a mechanism of drought tolerance characterised by the maintenance of green stems and upper leaves when water is limiting during grain filling. For the last two decades, sorghum breeders have used 'stay green' traits for indirect selection for drought resistance, although the physiological mechanism and genetic control of this trait are not well understood (Tao *et al.*, 2000).

Under normal field conditions, leaves of many typical sorghum lines senesce after grain maturity. Some genotypes, however, not only remain green (Duncan *et al.*, 1981), but also contain significantly more carbohydrates in the stem at all stages of maturity, more cytokinins and higher grain weight than senescent genotypes (McBee, 1984). 'Stay green' also reduces lodging in sorghum, (Henzell *et al.*, 1984) and there is an association with resistance to stem rots (Rosenow, 1984), suggesting that 'stay green' leaves, remain

photosynthetically active (Thomas and Howarth, 2000). Increased accumulation of soluble sugars in the 'stay green' type appeared to be associated with a greater functional leaf area during grain filling, thereby reducing their dependence on stored assimilates from the stem to fill the grain (Duncan, 1984).

Selection for the 'stay green' phenotype in sorghum has been conducted in multi-environment tests for developing superior drought resistant genotypes (Henzell *et al.*, 1997). The 'stay green' phenotype is only expressed in sorghum in tests in which terminal stress occurred and, therefore, neither the efficiency nor the reliability of selection is high when only conventional breeding approaches are used for selection. Quantitative trait (QTL) analysis and marker-assisted selection have greatly increased the efficiency of the selection of the 'stay green' trait. Recently, QTLs and markers linked to the 'stay green' trait in sorghum have been identified (Crasta *et al.*, 1999; Tao *et al.*, 2000) with four QTLs explained more than 50% of the phenotypic variation (Xu *et al.*, 2000). This approach will undoubtedly have great impact in the future, opening the way to an understanding of the molecular biology of drought-induced senescence.

In the absence of any physiological analyses of the trait in sorghum, it is difficult to classify the sorghum 'stay green' phenotype according to Fig. 10. However, because an increase in cytokinins reduced the rate of loss of both chlorophyll and photosynthesis in senescing wheat seedlings (Wittenbach, 1977), producing a type B 'stay green' phenotype, it is possible to suggest that some 'stay green' lines of sorghum may be type B (Thomas and Smart, 1993).

1.10. Context and aims of the project

Plant breeders have dramatically increased the potential yield of wheat and rice by creating short, "dwarf" varieties with strong stalks that hold a larger grain. This has increased the contribution of the grain to the total plant mass (i.e. harvest index) to about 50%. However, dwarfing did not result in increased yield in maize, because the shorter plants resulted too much shading. Maize breeders, therefore, took a different approach (i.e. 'stay green'). Furthermore, many scientists have argued that the yields of many cereals have physical limits, and that breeders and farmer may be nearing them in rice and in wheat. Nevertheless, the projected increase in global population, which will be accompained by an increase in demand for cereals, has led some crop scientists to create a new model of plant (i.e. ideotype) in order to meet the projected demand.

When the work described in this thesis was begun, the effects of extension of photosynthetic capacity on yield production in wheat were not known.

The principal aim of this project was, therefore, to develop a type of 'stay green' durum wheat that could be exploited in breeding programmes in order to increase the grain yield in this important crop.

In order to meet this projected aim, the main objectives of this project were:

1) To select and to characterise functional 'stay green' mutants in durum wheat.

2) To determine the physiological effects of these mutation.

3) To undertake a preliminary study on source-sink relationships in the mutants.

4) To understand the molecular and genetic bases of the mutations.

Chapter 2

Materials and Methods

2.1. Plant materials and mutagenesis

About 20,000 seeds of *Triticum durum* Desf. (cv Trinakria) were mutagenised either for 2h in 0.01 mM sodium azide, pH 3, at 25 °C (10,000 seeds) or in 0.3 M of ethylmethane sulphonate (10,000 seeds). Mutagenized seeds were then sown in the field [at the Experimental Institute for Cereal Crops (ISC) Foggia, Italy] and grown to maturity. One seed of each of the 20,000 plants (M_2 lines) was sown in the field and the plants selfed for two generations (M_5 lines). Four independent mutants were chosen for their similar timing of flowering but delayed timing of senescence and individual M_5 plants were grown in the field. Further experiments were performed on these lines.

2.1.1. *In vivo* experiments

Physiological characterisation was carried out in a controlled-environment glasshouse in IACR-Rothamsted, Harpenden, UK.

Plants of *Triticum durum* Desf. (cv Trinakria), and of each of the four mutants analysed were grown in 8 inch diameters pots filled with 75% L&P fine-grade peat, 12% sterilised loam, 3% medium grade vermiculite, 10% 6 mm screened, lime-free grift, 3.5 kg Osmocote *per* m^3 (slow-release fertiliser, 15-11-13 NPK plus micronutrients) 0.5 kg PG mix *per* m^3 (14-16-18 NPK granular fertilise plus micronutrients) in glasshouse cubicles under irradiance of ca. 750 μm $s^{-1}m^{-2}$, supplied by 400W sodium lamps with a 16 h light period, at 18-20 °C under lighting and 14-16 °C during darkness and 50-70 % relative humidity.

2.1.2. Gas exchange measurements

Leaf gas exchange measurements were carried out, once weekly, on attached flag leaves from flowering until full senescence from eight different plants, from both, 'stay green' mutants and parental plants. Rates from CO_2 exchange were measured on intact leaves under 800 μmol m^{-2} s^{-1} PAR. Chambers had forced ventilation, and the temperatures were regulated to maintain the leaves at 20 ± 1 °C. All measurements were made at ambient

CO_2 concentration (~350 μmol mol^{-1}) in a multi-chamber gas-exchange system with automatic data collection and analysis, using a Ciras 1 gas analyser (PPSystem).
Carbon dioxide exchange was then used to calculate photosynthetic rate, stomatal conductance and sub-stomatal CO_2 concentration.

2.1.3. Chlorophyll contents measurements

The relative chlorophyll contents was determined non-destructively on the same flag leaves using a hand–held chlorophyll meter (SPAD-502, Minolta, UK.). Each value was obtained by averaging readings from 10 sampling positions on the leaf of individual plants.

2.1.4. Fluorescence

PS II chlorophyll fluorescence was measured on flag leaves with a modulated meter (OSLOG-100), at room temperature. Leaves, attached to the plant, were enclosed in gas exchange chambers specially designed for simultaneous measurement of gas exchange and chl *a* fluorescence. To ensure maximun oxidation of QA and full dissipation of the transthylakoid proton gradient, leaf were dark-adapted for at least 40 min.
The minimal fluorescence level (F_0) with all the PS II reaction centers open was determinated using the measuring modulated light. The maximum fluorescence level (Fm) with all PS II reaction centers closed was determined by a two-second saturating light pulse (3000 μmol photons $m^{-2}s^{-1}$ in the range from 350 to 700 nm) provided by a 35 W lamp, in dark adapted leaves.
The *Fv/Fm* ratio, which measures the quantum yield of the photochemistry of PS II centres, was calculated as ($Fm-F_0$)/Fm (Genty et al., 1989)

2.1.5. Extraction and determination of total chlorophyll

Leaf discs (ca. 10 mm diameter) were excised in triplicate from different areas of each leaf, immediately frozen in liquid nitrogen and stored at -80°C. For chlorophyll extraction, samples were ground independently in 1 ml of cold 80% (v/v) acetone and

centrifuged at 11,000*x*g for 15 minutes at 4°C. This step was repeated two times with re-homogenisation of the pellet and the combined supernatants, diluted tenfold in 100% acetone, were used for chlorophyll a and b determination (Buchanan-Wollaston and Ainsworth, 1997). Chlorophyll a and b concentrations were determined immediately by measuring the absorbance at 663 nm and 646 nm.

Chlorophyll a and b concentrations were also calculated by the equation of Hill (Hill *et al.*, 1985) as follow:

$$\mathbf{Chl_a}\ (\mu g/ml) = 12.25\ A_{663} - 2.55\ A_{646}$$

$$\mathbf{Chl_b}\ (\mu g/ml) = 18.29\ A_{646} - 4.58\ A_{663}$$

2.1.6. Induction of senescence on detached leaves

For the induction of artificial senescence, plants were grown in 25 cm pots in a controlled greenhouse in Long Ashton Research Station (LARS, Long Ashton, Bristol, UK). The soil mixture was 6 parts shredded sterilised loam : 4 parts peat : 2 parts cornish grift and 3.5 kg Osmocote per m^3 (slow-release fertiliser, 15-11-13 NPK plus micronutrients) and the growth conditions were a irradiance of ca. 750 $\mu m\ s^{-1}m^{-2}$, (supplied by 400W sodium lamps) with a 16 h light period at 20-25 °C, and 14-16 °C during darkness and 50-70 % relative humidity. After booting, the fifth leaves of both parental and mutants plants, were excised, placed in 500 ml beakers filled with tap water and incubated in the permanent dark at ca. 20 °C for six days (Vincentini *et al.* 1995). The beakers, wrapped in parafilm and aluminium foil, were opened for inspection and sampling every two days.

Before any experiments, the leaf tips and the basal part which had been immersed in water were removed and discarded.

Three replicate samples for each stage were taken for the chlorophyll extraction and determination (Section 2.1.4.),

2.1.7. Mean weight of 1000 seeds

1000 mature seeds harvested from both parental and mutants plant were counted and mean weights calculated.

2.1.8. Extraction of total proteins from wheat seeds

Wheat grain was ground using a Maig mill to 0.2mm particle size and a modified Osborne procedure was used to extract proteins (Shewry *et al.*, 1983). The seed proteins were extracted by stirring in a series of solvents at 20°C with 10ml of solvent /g flour. Each extraction was carried out for 1 h as follows:

1. The samples were stirred with water-saturated butan-1-ol to remove lipids. This step was then repeated.
2. The supernatant was discarded and the pellet stirred with 0.5M NaCl to extract the salt-soluble proteins (albumins and globulins). This step was then repeated.
3. The pellet was re-homogenised and washed with distilled water to remove residual NaCl.
4. 50% (v/v) Propan-1-ol containing 2% (v/v) 2-mercaptoethanol and 1% (v/v) acetic acid was used to extract the prolamins fraction. This step was repeated twice.

The supernatants were separated by centrifugation for 20 min at 20,000*xg* and treated as follows:

1. Supernatants from steps 2 and 3 were combined and dialysed against several changes of distilled water.
2. Supernatants from point 4 were combined and the prolamins recovered after precipitation, by dialysis against distilled water.

2.1.9 Total Nitrogen (N)

The total nitrogen contents of milled wheat was determined by Kjeldahl analysis (Institue of Brewing Recommended Methods of Analysis (1991) method 1.5 and 2.9). A Tecator Kjeltec system (Hoganas, Sweden) was used. Duplicate samples (0.5-1g) were accurately

weighed onto nitrogen free paper, and placed in a digestion tube. Two tablets of Kjeltab catalyst and concentrated sulphuric acid (2 ml) were added to each tube. The tubes were placed in a heating block and heated at 150°C for 1.5 hours. The temperature was then gradually increased to 350°C until digestion was complete (approximately 3 hours). The tubes were then cooled and diluted with distilled water (50ml). After cooling, the tubes were placed in a Kjeltec 1002 distillation unit. Sodium hydroxide [40% (w/v), approximately 100ml] was dispensed into the tube and the distillate collected in a conical flask containing boric acid indicator [4% (w/v) 25ml]. Approximately 150ml was collected and titrated against hydrochloric acid (0.1M). Total nitrogen was calculated as follows:

$$\textbf{Total nitrogen (\%)} = \frac{\text{Titre x } 14.008}{\text{W x DM}}$$

Where:- Titre = sample titre - blank titre

W = sample weight (g)

DM = sample dry matter content (%)

2.1.9.1 Dry weight

The dry weight of flour samples was determined by drying in an oven at 110 °C for 24 h.

2.1.9.2 Total soluble nitrogen

The nitrogen contents of the prolamin and albumin/globulin fractions were determined by the Kjeldahl method (see total nitrogen). Samples (20ml) were digested as described above and total soluble nitrogen was calculated as follows:

$$\textbf{Total nitrogen (\%)} = \frac{\text{Titre x } 14.008}{\text{DM}}$$

Where:- Titre = sample titre - blank titre

DM = sample dry matter content (%)

2.1.10 SE-HPLC analyses of wheat seed proteins

Size – exclusion HPLC (SE-HPLC) has been extensively used for the study of cereal storage proteins, particularly in wheat. This methodology, which accurately separates the three main classes of wheat storage proteins : glutenins, gliadins and albuminis/globulins was performed on flour milled (Section 3.1) from randomly selected grains as follow:

-The total unreduced proteins from 10 mg flour samples were extracted in 1 ml of 50 mM sodium-phosphate buffer (pH 6.9.) containing 0.5% SDS and the samples were placed in a sonic bath for 30 minutes. The samples were then centrifuged at 20,000 x g and the supernatants were placed in an autosampler vial (Chromacol Ltd). 50 µl of the supernatant was loaded onto a TSK Gel 3000SW column (Phenomenex). The proteins were then separated by a 50%/50% gradient of water (filtered by 2 µm nylon membrane filters, Whatman) added with 0.07% trifluoroacetic acid (TFA) [Aldrich] and Acetonitrile (ACN) [BDH] added with 0.05% TFA.

A standard 30 minutes run (at flow rate of 0.5 ml per minute) was used.

2.1.11 Embryo weight

After determination of the dry weight of seeds (Section 3.2.1), the embryo was accurately eliminated and the embryo weight was calculated as follow:

Embryo weight (mg): $S_0 - S_1$

were S_0 = whole seed and S_1 = seeds without embryo

2.1.12. Differential Display of mRNAs

2.1.12.1. General introduction

Understanding how gene expression in cells is regulated in time and space is of great importance and constitutes one of the major objectives of research in biology. The methods most widely used to distinguish mRNAs expressed in different cell types or in cells grown under different conditions are based on subtractive or differential hybridisation. Recently, a powerful new technique has been developed. This is based on the assumption that virtually every mRNA expressed in a cell can be detected on gels by reverse trancription (RT), followed by polymerase chain reaction (PCR) amplification (Liang and Pardee, 1992, 1995) using arbitrary oligonucleotides that have a statistical chance of annealing to a corresponding sequence in an mRNA population. This novel method has undergone several modifications and has finally been designated "differential display reverse transcription – polymerase chain reaction" (DDRT-PCR; Bauer *et al.* 1993). DDRT-PCR has proved to be highly effective in identifying sequences that are differentially expressed in various cell types and, more importantly, in detecting genes coding for regulatory proteins (Liang *et al.*, 1992). One of the most remarkable advantages of this method is the relatively small amount of RNA that can be used compared with the amount required for subtractive and differential hybridization techniques.

An outline of the overall **DDRT-PCR** procedure is shown in **Fig. 2.**

Briefly, after reverse transcription of total RNA or messenger RNA, amplification of cDNA is achieved by PCR, followed by separation on sequencing gels. When a differentially expressed mRNA is identified, the corresponding cDNA can be eluted from the band excised from the gel, reamplified, and cloned in an appropriate plasmid vector. Effective differential gene expression must be confirmed by RNA slot blot and northern blot analysis before determing the sequence of the cloned gene. Because cDNAs cloned by DDRT-PCR are generally short (150-400bp) and represent the 3' region of the gene, the isolation of longer cDNAs or genes from libraries, is often necessary before searching for putative gene functions in sequence date bases.

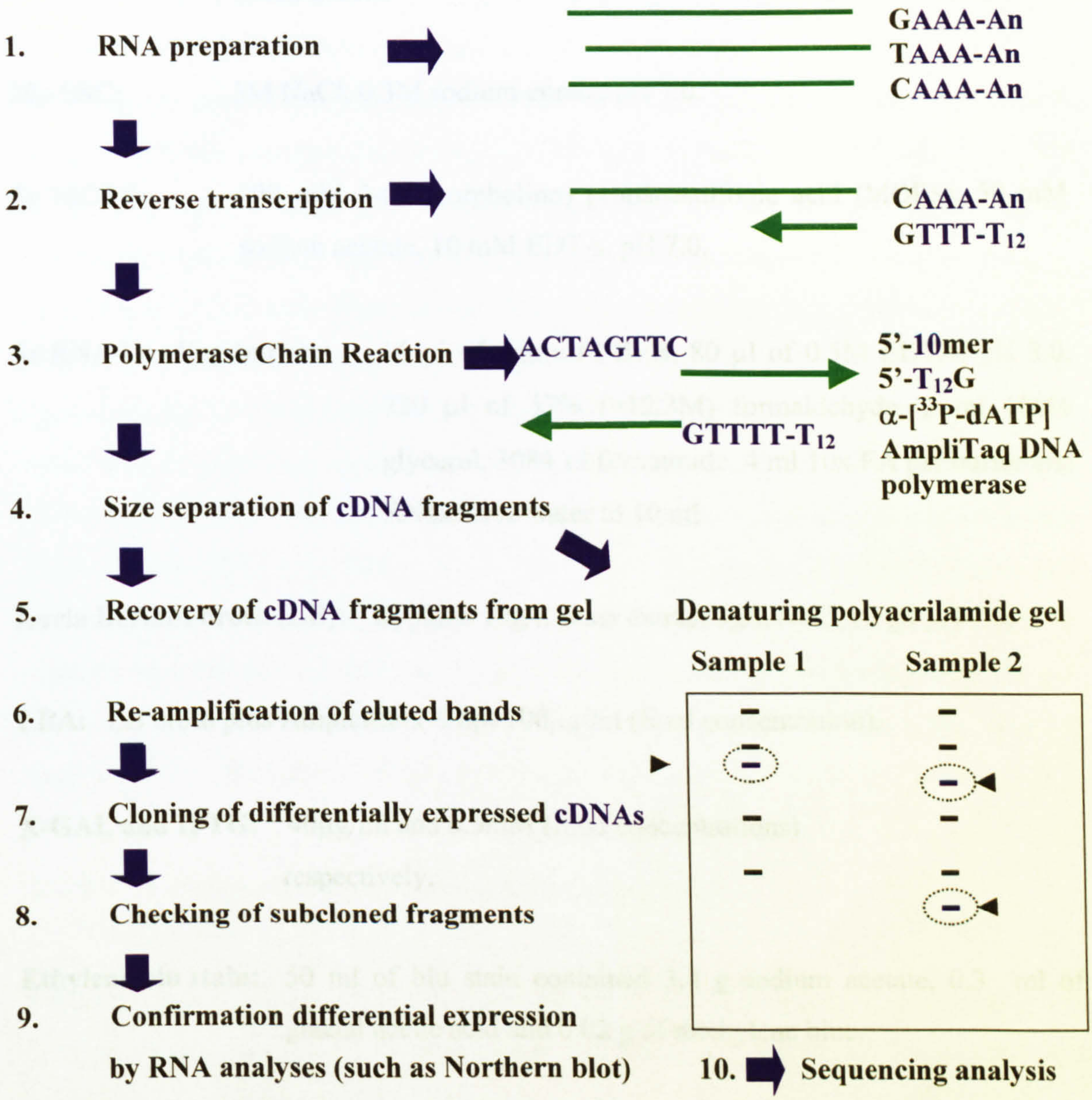


Fig. 2. Outline of DDRT-PCR.

DDRT – PCR involves reverse transcription of RNA (**2**) followed by PCR amplification (**3**), segregation of amplified cDNAs on acrylamide gel (**4**), cloning (**7**) and confirmation of differentially expressed fragments (**9**).

Each step is described in materials and methods section.

4.1.2. **2.1.12.2. Buffer and media used in the differential display technique are listed below.**

20x SSC: 3M NaCl, 0.3M sodium citrate, pH 7.0.

5x MOPS: 200 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0.

5x RNA Loading buffer: 16 µl of saturated BFB, 80 µl of 0.5M EDTA, pH 8.0, 720 µl of 37% (=12.3M) formaldehyde, 2 ml 100% glycerol, 3084 µl formamide, 4 ml 10x FA gel buffer and RNase-free water to 10 ml.

Luria Bertani broth (LB): tryptone 10g/l, yeast extract 5g/l, NaCl 10 g/l pH 7.0.

LBA: LB broth plus Ampicillin (Amp) 100µg/ml (final concentration).

X-GAL and IPTG: 40µg/ml and 0.5mM (final concentrations) respectively.

Ethylene blu stain: 50 ml of blu stain contained 3.4 g sodium acetate, 0.3 ml of glacial acetic acid and 0.02 g of methylene blue.

1x TAE Buffer: 40 mM Tris/acetate, 1mM EDTA.

Bacterial strain: JM 109; **genotype**: F' [*tra* D36 *pro*AB$^+$ *lac*I^q*lac*ZΔM15] *end*A1 *rec*A1 *hsd*R17 (rk$^-$,rk$^+$9 *sup*E44 *thi gyr*A96 relA1 Δ(*lac-pro*AB).

2.1.12.3 Differential display -Total RNA extraction

Total RNA was isolated from flag leaves of control and mutants plants using the Qiagen Rneasy Mini Kit (QIAGEN) as follows:

-samples were ground to a fine poweder under liquid nitrogen using a mortar and pestle. The ground tissue was then transferred to a tube and six hundred µl of Buffer RLT was added. The samples were then vortexed vigorously and the lysates applied to the QIAshredder spin columns (kit supplied) placed in 2ml collection tubes and centrifuged for 2 min at 13,000 rpm. Flow-through fractions from the QIAshredder columns were tranferred to new tubes without disturbing the cell debris pellet in the collection tubes. Approximately 0.5 volumes of ethanol (96-100% v/v) were added to each cleared lysate, mixed well by pipetting, applied onto Rneasy mini spin columns (pink) placed in 2ml collection tubes and centrifuged for 1 min at 13,000 rpm. Flow-through fractions were discarded and 700 µl of Buffer RW1 were pipetted onto the Rneasy columns and centrifuged for 1 min at 13,000 rpm. This step was repeated twice before the Rneasy columns were transferred into new 1.5ml collection tubes. Fifty microliters of RNAse-free water was pipetted directly onto the Rneasy membranes and the samples centrifuged for 1 min at 13,000 rpm to elute the RNA.

2.1.12.4. Differential display -Integrity of total RNA

Before mRNA purification, the integrity and size distribution of total RNA was checked by denaturing – agarose gel electrophoresis and ethidium bromide (EtBr) staining. A 5 µl aliquot of the total RNA was electrophoresed on a 1.2% (w/v) agarose gel with 0.1 mg ml^{-1} EtBr in 1x MOPS. If the ribosomal RNAs species appeared as sharp bands on the stained gel, the fraction was subsequently used for mRNA purification.

2.1.12.5. Differential display - mRNA purification

Purification of poly A^+ mRNA from total RNA was carried out using the Qiagen Oligotext ™ mRNA Kit (QIAGEN Ltd, UK) as follows:

250 µl of 2 x Binding Buffer and 15 µl of Oligotex Suspension were added to 250 µl of total RNA. Mixtures were incubated for 3 min at 65 °C, followed by an incubation for 10 min at room temperature, then centrifuged for 2 min at 13,000 rpm and the supernatants removed. The pellets (Oligotex resin containing the mRNA) were resuspended in 400 µl of Wash Buffer (OW2) and pipetted onto spin columns. This step was repeated twice before the mRNA samples were eluted with 50 µl of RNAase-free water.

2.1.12.6. Differential display - Quantification of mRNA

The concentration and purity of RNA was determined by UV spectrophotometry. Five microliter of mRNA in water was diluted in 995 µl sterile distilled water (SDW) in a clean quartz cuvette (1cm path length) previously treated with 0.1 M NaOH, 1 mM EDTA followed by washing with water. A scan of absorbance readings was taken between wavelengths of 210 to 330 nm against a SDW blank. The reading at 260 nm allowed calculation of the concentration of nucleic acid. An absorbance of 1 at 260 nm (A_{260}) corresponds approximately to 40 µg ml^{-1} of single stranded RNA (50 µg ml^{-1} of double stranded DNA). Hence, the concentration of mRNA in the cuvette was calculated using the following formula: mRNA (mg ml^{-1}) = 40 µg ml^{-1} x A_{260} x dilution factor (50 µg ml^{-1} for DNA samples).

The ratio between the readings taken at 260 nm and 280 nm (A_{260}/A_{280}) has provided an estimation of the putity of RNA. In each cases the ratio was about 2, indicated a good quality of the RNA.

RNA samples were diluted to a final concentration of 0.1 µg/µl in distillated water treated with 0.1% of diethyl pyrocarbonate (DEPC) and used directly for cDNA synthesis or stored as an ethanol/acetate precipitate at –80°C.

2.1.12.7. Differential display - cDNA synthesis

Two microliter of mRNA from control plants and a mix of mRNA from all the four mutants (equal amount of each - 2µl total mRNA) was used as template for first strand cDNA synthesis. Two cDNA preparations were made from each mRNA preparation and

PCR was performed in parallel on these two preparations. Differentially expressed bands were accepted as positives only if they appeared in both reactions.

cDNA for DDRT-PCR was synthesised using the Bulk First-Strand reaction Mix (Amersham Pharmacia) as follows:

-2µl of mRNA sample (0.1 µg/µl) were placed in a microcentrifuge tube and RNAse-free water was added to bring the mRNA volume to 8µl. Samples were heated at 70°C for 10 min, quickly chilled on ice and briefly centrifuged. Five microliters of Bulk First-Strand cDNA Reaction Mix, 1 µl of DDT Solution and 1 µl of the anchor primer (10µM) were added to the samples. The mixtures were gently pipetted up and down several times to mix and then incubated at 37°C for 1 hour. Finally, the completed first-strand reaction was heated at 90°C (in a heating block) for 5 min to denature the RNA-cDNA duplex and inactive the reverse transcriptase, and chilled immediately on ice.

The anchor primers used were $T_{12}A$, $T_{12}G$, $T_{12}C$, T_{13}.

2.1.12.8. Differential display – PCR amplification

2 µl of the cDNA preparation was used in a standard PCR reaction with the appropriate dT_{12} primer and a random 10-mer oligonucleotide (Operon 10-mer Kits; **Tab. 2.1.**). A reaction mixture for each primer set combination was set up. In a total volume of 20 µl, 2.0 µl 10x PCR buffer, 1.6 µl dNTP mix (25 µM), 2.0 µl $T_{12}M$ (10µM with M=G,A,T,C), 2.0 µl 10-mer primers (2.5µM), 2.0 µl cDNA, 0.3 µl dATP α ^{33}P (Amersham), 0.2 µl ampliTaq (5U/µl) (Promega) and 8.3 µl H_2O were added. After the mixture was gently mixed the sequencing reaction were performed in a Perkin Elmer 9600 thermocycler. Thermocycling conditions were as follows: 40 cycles with denaturation at 95 °C for 1 min, annealing at 42 °C for 2 min, and extension at 72 °C for 30 seconds.

2.1.12.9. Differential display –Size separation of cDNA fragments

PCR-amplified cDNA fragments were separated on denaturing 6% (w/v) polyacrylamide gels in 1x Tris-borate buffer, pH 8 (Liang and Pardee, 1992). The gel was blotted onto Whatman 3MM paper, dried without fixing and exposed for 48 hours.

2.1.12.10. Differential display – Elution of cDNA fragments and re-amplification of eluted bands

Differentially expressed bands were accepted as positives only if they appeared in both reactions (see fig. 4.1.). Differential display bands were excised with a sterile razor blade from the non-fixed polyacrylamide gel, soaked in 100 µl dH_2O for 30', boiled for 15', spun in a microcentrifuge at 15,000 rpm for 15 min, dried in a speed vacuum and resuspended in 10 µl of water. A 2 µl aliquot was used to re-amplify the cDNA fragments in a 20 µl reaction mixture, under the same conditions used for the DDRT-PCR, except that the dNTP concentration used was 250 µM instead of 25 µM and no isotope was added.

2.1.12.11. Differential display –Purification of of differentially expressed cDNAs

An 10 µl aliquot of the resulting PCR product was electrophoresed on a 1.8% (w/v) low melting point agarose gel with 0.1 mg ml^{-1} EtBr in 1x TAE buffer separated at 50-60 mA for 1.5 hours. After visualisation under UV light, the gel slices containing the desired bands were removed and placed in a pre-weighed 2-ml Eppendorf tube. The gel slice in the Eppendorf tube was then weighed to determine the weight of the gel slice. Three volumes of buffer QX1 were added to 1 volume of gel containing the desired bands. QIAEX II silica particles were resuspended by vortexing for 30 s and 30 µl added immediately to the tube. To solubilise the gel and bind DNA to the particles, the mixture was incubated at 50 °C for 10 min, vortexing every 2 min to keep the silica particles in suspension. After incubation, the samples were centrifuged at 13,000 rpm and the supernatant removed. To wash the residual agarose the pellet was resuspended in 500 µl buffer QX1 and centrifuged for 30 s and the supernatant removed. To remove residual salt contaminants, the pellet was washed twice with 500 µl buffer PE. After the last wash, the supernatant was removed and the pellet air-dried for 30 min until white. To elute the DNA the silica particles were resuspended in 20 µl SDW, briefly vortexing and incubated at 50 °C for 5 min. The mixture was centrifuged for 30 s and the supernatant containing the purified DNA collected. The approximate DNA yield after extraction was determined by agarose gel electrophoresis as described in Section 4.1.5.

2.1.12.12. Differential display –Ligation of differentially expressed cDNAs

To clone differentially expressed cDNAs, a pGEM-T Easy Vector (Promega) was used. The presence of 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of PCR products into the plasmid. Furthermore, because the PCR product is inserted in the coding region of the enzyme β-galactosidase, it allows recombinant clones to be directly identified by colour screening on indicator plates. The recombinant DNA clones (vector with DNA fragments) will appear as white colony, while the non recombinant ones (vector without DNA fragment) will appear as blue colony. A 10 µl ligation reaction mixture was set up either for each cDNA fragments or for a control reaction. This consisted of 3.0 U T4 DNA ligase (Promega), 2x Rapid Ligation Buffer and a 1:3 ratio of pGEM-T Easy Vector : PCR product. The mixtures were incubated overnight at 4°C. To isolate the DNA ligation products, 40 µl of water plus 110 µl of 100% ethanol plus 5 µl of 3M NaOAc pH 5.2, were added.

The mixtures were centrifuged at 13,000 rpm for 30 min at 4 °C, the supernatant discarded and 200 µl of 70% (v/v) aq. ethanol added to the pellet. The DNA was then centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were discarded and the pellets containing the DNA were dried in a speed vacuum and then dissolved in 7 µl of distilled sterile water. After ligation, transformation of the ligated cDNA was performed by either electroporation or by $CaCl_2$.

2.1.12.13. Differential display –Transformation of E. coli cells by electroporation

For each ligation reaction, 3 µl DNA was added to 40 µl competent *E.coli* cells (Invitrogen) in cuvettes that had been pre-chilled at –20°C for 1h. The bacteria /DNA mixtures were electroporated at 200 Ω and 2.5 KV. Immediately after electroporation, 400 µl LB Luria-Bertani medium was added and mixtures were transferred to 10 ml polypropylene tubes. Mixtures were incubated at 37 °C with shaking at 200 rpm for 1 h. Thereafter, 100 µl of the mixtures were spread onto LBA plates containing LB medium plus 100 µg ml^{-1} ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The plates were then incubated overnight at 37 °C.

2.1.12.14. Differential display –Transformation of E. coli cells by $CaCl_2$

For each ligation reaction, 5 µl of 0.5 M 2-mercaptoethanol were added to 100 µl of Ultracomp[tm] *E.coli* (Invitrogen) in tubes that have been pre-chilled at –20 °C and the cells were incubated on ice for 10 min. After 5 µl of DNA was added, the solutions were gently mixed and incubated on ice for 30 min. The cells were warmed in a 42 °C water bath for 45 seconds and then chilled on ice for 2 min. After addition of 900 µl of SOC medium, the mixtures were incubated at 37 °C with moderate agitation (225 rpm) for 1 h and 100 µl of the mixtures were spread onto LBA plates plus IPTG and X-GAL. The plates were then incubated overnight at 37 °C.

2.1.12.15. Differential display – Checking subcloned fragments

For screening positive clones, single, white recombinant bacterial colonies were randomly picked and inoculated into 10 ml liquid LBA medium. Cultures were grown for 4 h at 37°C with shaking. Plasmid DNA was isolated using the Wizard *Plus* SV Minipreps DNA purification System (Promega) as follow:

-1.5ml of bacterial culture was centrifuged in a 1.5ml Eppendorf tube at 15,000 rpm at room temperature for 10 min. The supernatant was discarded and another 1.5 ml of bacterial culture was pelleted into the same tube. After the second centrifugation, the pellet was resuspended in 250 µl resuspension solution by vortexing, 250 µl cell lysis solution was added to each sample and the tubes immediately inverted five times to mix. A 10 µl alkaline protease solution was then added and the tubes immediately inverted five times to mix. After incubating the samples at room temperature for 5 minutes, 350 µl neutralisation buffer was added. The tubes were gently mixed by inverting a few times and subsequently centrifuged at 13,000 rpm for 10 min at room temperature. To centrifuge, a spin column (supplied in the kit) was placed in an unlidded Eppendorf tube and the supernatant from the centrifuged samples was pipetted onto the column. The spin columns containing the samples were then centrifuged at 13,000 for 1 min to drain off the flow-through fraction. Centrifugation of the spin columns was repeated twice after applying 750 µl and 250 µl of wash buffer, respectively. After elution of the wash buffer,

the columns were spun again briefly to remove the excess liquid. To elute the DNA, the columns were placed into clean 1.5 ml Eppendorf tubes (without lids). Fifteen microliters of nuclease-free water were applied and the columns were left to stands for 1 min to re-dissolve the DNA. They were then spun at 13,000 for 1 min at room temperature to elute the DNA.

To confirm and to identify positive clones, one restriction endonuclease digests was set up for each recombinant DNA sample to isolate the differentially expressed fragment. A 20 µl reaction mix was set up for each sample which consisted of 1µg DNA, 10 U of restriction enzyme (*EcoR* I), and 1x of enzyme buffer H from Promega. Digestion was carried out at 37°C for 1 h and the digestion products were then analysed by electrophoresis using 1.2% (w/v) agarose gel with 0.1 µg ml^{-1} EtBr in 1x TBE buffer. Gels were run at 70mA for about 1.5 h.

A 1 Kb ladder was used as molecular marker to check the size of the fragments.

2.1.12.16. Differential display –Reverse northern blotting analysis

Aliquots of the re-amplified fragments were quantified spectrophotometrically (A_{260}) and the same amount of each fragment was used. The fragments where then run in duplicate on the same 1.6 % (w/v) agarose gel with 0.1 µg ml^{-1} EtBr and the gel was blotted onto the nylon membrane (Amershan International, UK). The membrane was then cut and the duplicate membranes were hybridised at 65 °C for 12 hours in a buffer containing 5 x SSC, 0.5 x SDS 50 x Denhardt's solution. cDNA preparations from senescing leaves of either control or mutant plants (section 4.1.6), were used as probes.

The differentially expressed fragments were re-cloned and used for northern blot analyses.

2.1.12.17. Differential display –DNA labeling and hybridisation

In order to obtain high specific activity a Prime-It II Random Primer Kit (Stratagene) was used to label the probes

A 10 µl solution of random oligonucleotide primers and 33 µl dH_2O were added to 1 µl (25 ng/µl) of DNA template. The reaction was then heated in a boiling water bath for 5 min, briefly centrifuged and chilled on ice. 10 µl of 5 x dATP buffer, 5 µl of of [α–^{32}P] dATP (3000 Ci/mmol, Amersham) and 1 µl of Exo(-) Klenow enzyme (5 U/µl), were added, the solution briefly mixed and incubated at 37 °C for at least 2 min. The reaction were then stopped by adding 2 µl of stop mix solution (kit suppllied) and the probes purified using a Nuc Trap probe purification Kit (Stratagene). Pre-hybridisation of the membranes was carried out at 65 °C for at least four hours in prehybridising solution containing 0.5 M Na_2HPO_4, 7 % SDS and 1mM EDTA. The probes were denatured at 100 °C for 10 ml, added to the solution and left to hybridise for 16 hours. The membranes were then washed under high stringency conditions (0.2 x SSC, 0.1 x SDS) and exposed to Amersham Hyperfilm-MS (Amersham International, UK) at –80 °C for 12 hours.

2.1.12.18. Differential display –Confirmation of Differential expression of cloned cDNA fragments

Northern blotting analysis was used to confirm that a cDNA band isolated from a differential display gel was a true positive and represented a gene differentially expressed in the system under study.

12 µg samples of total RNA from senescent leaves from control and mutant plants were separated by electrophoresis under denaturing conditions on 1.3% (w/v) agarose gel in MOPS 1x buffer. The melted agarose was cooled to a 65 °C in a waterbath and 1.8ml of 37% (12.3M) formaldehyde and 1 ml of EtBr (10 µg ml^{-1}) was added. The gel was pre-run for 30 min in 1x MOPS buffer before samples were loaded. The RNA samples were mixed with one volume of 5x RNA loading buffer, denatured at 65°C for 10 min, chilled on ice and loaded on the agarose gel.

The gel was run for 2 h at 75 V and the RNA was transferred to Hybond N membranes (Amersham International, UK) using 20 x SSC as transfer buffer. After blotting the membrane and RNA were fixed by UV cross linking for 2 min.

To check the integrity and amount of total RNA loaded per lane, methylene blue was used to stain the membrane for 2 min and DEPC-treated water was used to destain the

background to see the rRNA. The stain was removed completely with several changes of prehybridising solution (0.25 M sodium phosphate, 7 % SDS pH 7.0) over 2 h.

2.1.12.19. Differential display –Pre-hybridisation and hybridisation of the membranes

Pre-hybridisation of the membranes was carried out at 65 °C for at least four hours in prehybridising solution containing 0.5 M Na_2HPO_4, 7 % SDS and 1mM EDTA. The probes were denatured at 100 °C for 10 ml, added to the solution and left to hybridise for 16 hours. The membranes were then washed under high stringency conditions (0.2 x SSC, 0.1 x SDS) and exposed to Amersham Hyperfilm-MS at –80 °C for either 24 or 72 hours.

2.1.12.20. Differential display –Sequencing of differentially expressed fragments

Plasmid DNA sequencing was carried out using the ABI PRISM dRhodamine Cycle Sequencing Ready reaction Kit supplied with a heat stable Amplitaq DNA polymerase, FS (Applied Biosystems, UK). Three sequencing reactions were set up for each cDNA. A reaction mix of 20 µl was prepared which consisted of 8 µl of Terminator Ready Reaction Mix containing DNA polymerase, 0.5 µg of template of DNA and 3.2 pmol of primer. A universal primer (pUC/M13 Forward and Reverse Sequencing Primer) was used for each reaction. Thermocycling conditions using the Perkin Elmer 9600 thermocycler were as follows: 25 cycles with denaturation at 96 °C for 30 s, annealing 50 °C for 15 s and extension at 60 °C for 4 min.

Sequencing products were recovered from the mixture by ethanol precipitation. Using 0.5 ml Eppendorf tubes, 50 µl 95 % (v/v) aq. ethanol and 2 µl 3 M NaOAc, pH 5.2, were mixed with 20 µl contents of the reaction tubes (excluding mineral oil). The mixtures were vortexed, placed on ice for 10 min and centrifuged at 13,000 rpm for 30 min. The DNA pellets were rinsed with 70% (v/v) aq. ethanol. The DNA pellets were dried under vacuum for 5 min and redissolved in 4 µl of a 5:1 mixture of deionised formamide and EDTA/Blue dextran loading buffer (25 mM EDTA, pH 8.0, containing 50 mg ml^{-1} Blue dextran). The tubes were vortexed and briefly centrifuged. Before loading the samples

were heated at 90 °C for 2 min to denature and were kept on ice until loaded. The samples were sequenced using an ABI 373 DNA Sequencing System (Applied Biosystem, UK).

NAME	5' 3'	NAME	5' 3'
OPA-1	CAGGCCCTTC	OPB-1	GTTTTCGTCC
OPA-2	TGCCGAGCTG	OPB-2	TGATCCCTGG
OPA-3	AGTCAGCCAC	OPB-3	CATCCCCCTG
OPA-4	AATCGGGCTG	OPB-4	GGACTGGAGT
OPA-5	AGGGGTCTTG	OPB-5	TGCGCCCTTC
OPA-6	GGTCCCTGAC	OPB-6	TGCTCTGCCC
OPA-7	GAAACGGGTG	OPB-7	GGTGACGCAG
OPA-8	GTGACGTAGG	OPB-8	GTCCACACGG
OPA-9	GGGTAACGCC	OPB-9	TGGGGGACCT
OPA-10	GTGCTCGCAG	OPB-10	CTGCTGGGAC
OPA-11	CAATCGCCGT	OPB-11	GTAGACCCGT
OPA-12	TCGGCGATAG	OPB-12	CCTTGACGCA
OPA-13	CAGCACCCAC	OPB-13	TTCCCCCGCT
OPA-14	TCTGTGCTGG	OPB-14	TCCGCTCTGG

Table 2.1. Examples of random primers used in the DDRT-PCR experiments.

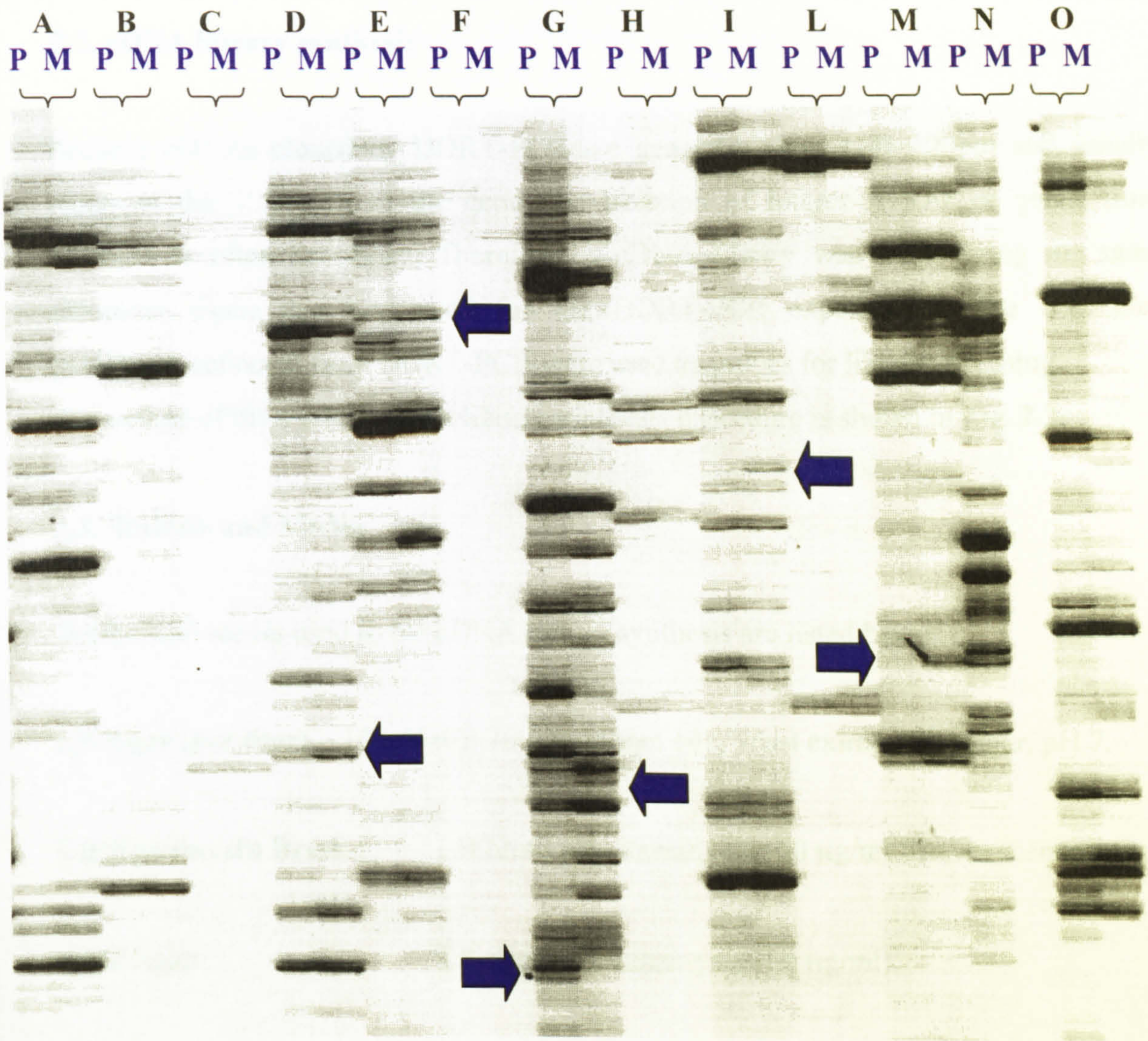


Fig.2.1. Denaturing gel electrophoresis of DDRT-PCR products obtained with different 3', 5' primer combination.

The arrows show putative differentially expressed mRNA. **M:** cDNA from mutants plants.

P: cDNA from control plants.

A,……**O:** 10-mer primers used for the DDRT-PCR experiments.

2.2. cDNA library synthesis

Because cDNAs cloned by DDRT-PCR are generally short (150-400bp) and usually represent the 3' region of the gene, the isolation of longer cDNAs or genes from libraries, is often necessary. Therefore, a cDNA library was made using the same senescent tissue that was used for the DDRT-PCR experiments. The fragments previously collected from DDRT-PCR were used as probes for library screening.

An outline of the overall cDNA library synthesis procedure is shown in **Fig. 2.2.**

2.3. Buffers and Media

Buffers and media used in the cDNA library synthesis are listed below.

LB Agar (per liter) 10 g NaCl; 10 g tryptone; 10 g yeast extract; 20 g agar, pH 7.

LB-Kanamycin Broth : LB broth plus kanamycin (50 μg/ml final concentration).

LBK Agar: LB agar plus kanamycin (50 μg/ml).

LB-Tetracycline Broth: LB broth plus tetracycline (12.5 μg/ml final concentration).

LBT Agar LB agar plus tetracycline (12.5 μg/ml).

NZY Broth (per liter): 5 g NaCl; 2 g $MgSO_4 \cdot 7H_2O$; 10 g NZ amine (casein hydrolysate).

NZY Agar : NZY Broth plus 0.15% (w/v) agar.

NZY Top Agar (per liter): NZY Broth plus 0.7% (w/v) agarose.

SM buffer (per liter): 5.8 g NaCl; 2 g $MgSO_4 \cdot 7H_2O$; 50.0 ml 1M Tris-HCL (pH 7.5); 5.0 ml 2% (w/v) gelatin.

10x STE buffer (per liter): 1M NaCl; 200 mM Tris-HCL (pH 7.5); 100 mM EDTA.

Column-Loading Dye 50% (v/v) glycerol; 10% (v/v) 10x STE buffer; 40% (w/v) saturated BPB.

10x MOPS Buffer 200 mM 3-[N-motpholino]propane-sulfonic acid (MOPS); 50 mM sodium acetate; 10 mM EDTA, pH 7.

2.3.1. Bacterial strains

Bacterial strains and growing media used for cDNA library synthesis are listed below.

Host strain	Genotype
SOLR strain	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacf^{q}ZΔM15 Tn 10 (Tet^r)]*
XL1-Blue MRF' strain	*E14$^-$ (McrA$^-$) Δ(mcrCB-hsdSMR-mrr) 171 sbcC recB recj uvrC umuC::Tn5 (Kan^r) lac gyrA96 relA1 thi-1 endA1 λ^r [F' proAB lacI^{q}ZΔM15] Su$^-$ (nonsuppressing)*

In each case, the medium used to grow bacterial cultures for titreing phage was LB supplied with 0.2% (w/v) maltose and 10 mM $MgSO_4$. The *ExAssist^{TM} interference – resistant* was used as Helper phage.

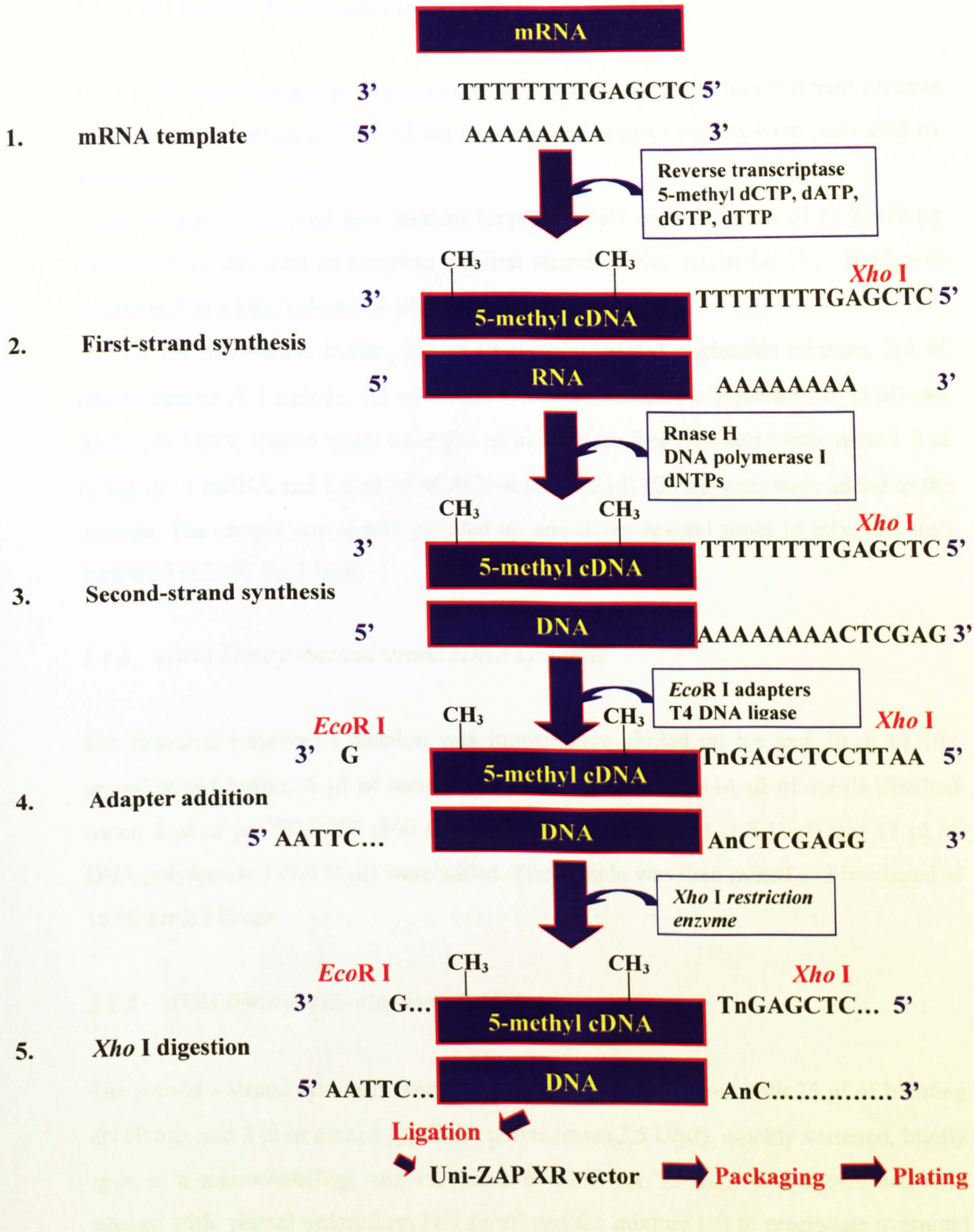


Fig.2.2. cDNA synthesis flow chart.

Each step is described in materials and methods.

2.4. cDNA library -First strand cDNA synthesis

Total RNA from flag leaves was extracted and purified from the four different mutants as described in Section 2.12.3. All the enzymes and relative buffers were purchased by Stratagene.

A mix of mRNA from all four mutants (approximately equal amounts of each ≈10 μg total mRNA) was used as template for first strand cDNA synthesis. The cDNA was synthesised in a total volume of 50μl, as follows:

-5μl of 10x first-strand buffer, 3μl of first-strand methyl nucleotide mixture, 2μl of linker –primer (1.4 μg/ml), 1μl of RNase Block Ribonuclease Inhibitor (40 U/μl) and 32.5 μl of DEPC-treated water were placed in a RNase-free tube and gently mixed. 5 μl (2 μg/μl) of mRNA and 1.5 μl of MMLV-RT (50 U/μl) (Stratagene) were added to the mixture. The sample was gently pipetted up and down several times to mix, and then incubated at 37°C for 1 hour.

2.4.1. cDNA library -Second strand cDNA synthesis

The first-strand synthesis reaction was immediately chilled on ice and 20 μl of 10x second-strand buffer, 6 μl of second-strand dNTP mixture, 114 μl of sterile distilled water, 2 μl of [α-^{32}P]dNTP (800 Ci/mmol), 2 μl of RNase H (1.5 U/μl) and 11 μl of DNA polymerase I (9.0 U/μl) were added. The sample was then mixed and incubated at 16 °C for 2.5 hours.

2.4.2. cDNA library –Blunting the cDNA termini

The second – strand synthesis reaction was placed on ice, mixed with 25 μl of blunting dNTP mix and 2 μl of cloned *Pfu* DNA polymerase (2.5 U/μl), quickly vortexed, briefly spun in a microcentrifuge and incubated at 72°C for 30 min. The cDNA was then washed with phenol-chloroform [1:1 (v/v)] and the mixture left to precipitate overnight at–20 °C.

2.4.3. *cDNA library –Ligation of the Eco*R I *adapters and phosphorylation of the Eco*R I *ends*

The second-strand synthesis reaction was spun at 4 °C for 60 min and the pellet washed with 500 ml of 70% (v/v) ethanol and lyophilised. The pellet was then resuspended in 9 µl of *Eco*R I adapters and incubated at 4 °C for at least 30 min. 1 µl of 10x ligase buffer, 1 µl of 10 mM rATP and 1 µl of T_4 DNA ligase (4 U/µl) were then added and the sample incubated at 8 °C overnight.

The ligase was then heat-inactivated by placing the tube in a 70 °C water bath for 30 min, the reaction cooled at room temperature for 5 min and the adapter ends phosphorylated by adding 1 µl of ligase buffer, 2 µl of 10 mM rATP, 6 µl of sterile water and 1 µl of T_4 polynucleotide kinase (10 U/µl). The mixture was incubated at 37 °C for 30 min and the kinase was then heat inactivated at 70 °C for 30 min.

2.4.4. *cDNA library –Digestion with Xho* I.

The enzymatic digestion was performed as follow:

-28 µl of *Xho* I buffer and 3 µl of *Xho* I (40 U/µl) were added to the sample and the reaction was incubated at 37 °C for 1.5 hours. The reaction was then mixed with 5 µl of 10x STE buffer and 125 µl of 100% (v/v) ethanol and precipitated at –20 °C overnight. The mixture was then spun in a microcentrifuge at 15,000 rpm at 4 °C for 60 min, the supernatant discarded, and the pellet dried and resuspended in 1x STE buffer.

The sample was then size-fractionated through a drip column containing Sepharose CL-2B following the instruction manual (Stratagene).

2.4.5. *cDNA library –Processing the cDNA fractions*

12 fractions (theoretically > 400 bp) were collected from the column and were separated on a 5% (w/v) non-denaturing acrylamide gel to assess the effectiveness of the size fractionation and to determine which fractions should be used for further analysis. Based on the gel results, 3 of the 12 fractions were used to remove contaminating proteins, especially kinase which could be carried over from previous steps in the synthesis. The

samples were therefore extracted once by adding an equal volume of phenol-chloroform [1:1 (v/v)], vortexed and spun in a microcentrifuge at 15,000 rpm at room temperature for 2 min. The upper aqueous layer was then transfered in a fresh tube, an equal volume of chloroform was added and the mixtures were spun in a microcentrifuge at 15,000 rpm at room temperature for 2 min. 100% (v/v) ethanol was then added and the samples left to precipitate overnight at –20°C. The samples were then spun at 4 °C for 60 min, the pellet washed with 200 µl of 80% (v/v) ethanol and dried. The cDNA from all the three samples was then resuspended in 5 µl of sterile water (total volume) and quantified spectrophotometrically (Section 4.1.6.).

2.4.6. cDNA library –Ligation of cDNA into the Uni-ZAP XR Vector

A 4.5 µl ligation reaction mixture was set up. This consisted of 1 µl of resuspended cDNA (0.1 ng μl^{-1}), 0.5 µl 10x ligase buffer, 0.5 µl of 10 mM rATP (pH 7.5), 1.0 µl of the UNI-ZAP XR vector (1 µg/µl), 1.5 µl of water and 0.5 µl of T_4 DNA ligase (4 U/µl). The mixture was then incubated overnight at 12°C and 1 µl was used to be packaged in a Gigapack III Gold packaging extract.

2.4.7. cDNA library –Packaging

The host strains (Section 4.2.2.) were streaked onto the appropriate agar plates (LBT or LBK) and incubated at 37 °C overnight. A single colony of XL1-Blue MRF'was then inoculated in the appropriate medium (Section 4.2.2.) and the cultures were grown at 37 °C until an OD_{600} of 1 (determined spectophotometrically). The cells were spun at 500xg for 10 min, the supernatant discarded and the cells gently resuspended with sterile 10 mM $MgSO_4$ and diluted to an OD_{600} of 0.5.

2 µl of ligated DNA (0.5 µg/µl) were then added to the packaging extracts which had previously been removed from the –80 °C freezer and placed on dry ice. The mixture was gently pipetted up and down and then incubated at 22 °C for 2 hours. 500 µl of SM buffer and 20 µl of chloroform were then added to the sample, gently mixed and briefly spun to sediment the debris.

The supernatant, containing the phage, was used for titre.

2.4.8. cDNA library –Plating and primary titre

For primary library titre three different dilutions were used as follows: 2 µl of the phage stock were added to 198 µl of SM buffer (=10^{-2} dilution) and then 20 µl of 10^{-2} dilution were added to 180 µl of SM buffer (=10^{-3} dilution). 20 µl of 10^{-3} dilution were then added to 180 µl of SM buffer (=10^{-4} dilution). 1 µl of each dilution was added to 200 µl of XL1-Blue MRF' cells in a 15 ml tube and the mixtures were incubated at 37 °C for 15 min to allow the phage to attach to the cells. 5 ml of NZY top agar (melted and cooled to 48-50 °C), 15 µl of 0.5M IPTG and 50 µl of X-gal (250 mg/ml) were then added and the mixtures immediately plated onto NZY agar plates. The plates were then allow to set for 10 min and incubated at 37 °C for at least 8 hours. Recombinant white plaques were counted and the titre of the phage in the cDNA library was calculated using the following equation:

$$\textbf{Titre (pfu/ml)} = \frac{[\text{Number of plaques (pfu) x dilution factor}] \text{ x total phage volume}}{\text{Volume plated}}$$

Because the titre of phage in the cDNA library will decrease with time (over a few years it may be reduced by 50%) amplification of the primary library is often necessary.

2.4.9. cDNA library –Amplifying the Uni-ZAP XR library

The primary library was amplified as follows:

-About 1.5 x 10^5 pfu were added to 2 ml host cells (Section 4.2.9.) in a 50 ml Falcon tube and the mixtures was incubated at 37 °C for 15 min to allow the phage to attach to the cells. 40 ml of NZY top agar (melted and cooled to 48-50 °C) was then added and the mixtures immediately plated onto 2 day old 24 x 24 cm NZY agar plates. The plates were then allowed to set for at least 30 min and incubated at 37 °C for 8 hours. This resulted in plaques that were well separated (being smaller than 2 mm) and the plates were therefore overlaid with 10 ml of SM buffer and stored at 4°C overnight with gentle

shaking (to allow the phage to diffuse into the SM buffer). The bacteriophage suspension was then recovered, pooled into a sterile polypropylene tube and chloroform added to final concentration of 5% (v/v).

After an incubation at room temperature for 15 min, the cell debris were removed by centrifugation at 500x*g* for 10 min and the supernatant was transferred to a new polypropylene tube. This step was repeated twice and then aliquots of the amplified library were stored in 0.3% (v/v) chloroform and 7% (v/v) DMSO at -80°C.

Dilutions and trites of the amplified library were calculated as described in Section 2.4.8.

2.4.10. cDNA library –Plating and lifting

The amplified library was plated at $5x10^5$ pfu/plate on 8 large 24 x 24 NZY agar plates (2 days old) as described in Section 4.2.10. After incubation for about 8 hours, the plates were chilled at 4 °C for 2 hours to prevent the NZY top agar from sticking to the membrane.

Lifting of amplified library was performed in duplicate as follows:

a nylon membrane was placed onto each NZY agar plate for 2 min to allow the transfer of the phage particles to the membrane and several pen marks were used to prick trough the membrane and agar for orientation. The membranes were then denatureted for 4 min in denaturation solution (1.5 M NaCl and 0.5 M NaOH), neutralised for 4 min in neutralisation solution (1.5 M NaCl and 0.5 M Tris-HCl, pH 8) and rinsed for an additional 4 min in 2x SSC. The membranes were then left to air dry for at least 10′ on Whatman 3MM paper and then baked at 80 °C for 30 min. The second membranes were allowed to transfer for 6 min and finally, the plates were stored at 4 °C until used.

2.4.11. cDNA library –Screening and in vivo *excision of the pBluescript phagemid from the UNI-ZAP XR vector*

The membranes were hybridised with a mix of differentially expressed fragments. Three rounds of screening were performed and the plaques of interest were removed from the agar plate as cores using sterile glass pipette and transferred to a sterile 15 ml tubes

containing 500 µl of SM buffer and 20 µl of chloroform. The tubes were then vortexed and incubated at room temperature for 2 hours. 250 µl of phage, 200 µl of fresh XL1-Blue MRF' cells and 1 µl of ExAssist helper phage were then added and the tubes were incubated at 37 °C for 15 min. The samples were then mixed with 3 ml of LB broth, incubated at 37 °C for 3 hours, heated at 65-70 °C for 20 min and spun at 1000 x *g* for 15 min. The supernatant was then transferred in a new tube and 10 µl of the phage supernatant were added to 200 µl of freshly grown SOLR cells (OD_{600} = 1.0). The samples were incubated at 37 °C for 15 min and 200 µl of the cell mixture were plated on LBA-agar plates (50 µg ml^{-1}) and incubated at 37 °C overnight.

2.4.12. cDNA library – Checking subcloned fragments

To confirm positive clones, the pBluescript phagemide containing the cloned DNA insert was extracted.

Restriction endonuclease digests was set up for each pBluescript phagemide to isolate the DNA insert. A 20 µl reaction mix was set up for each sample which consisted of 1µg DNA, 10 U of restriction enzymes (*EcoR* I and *Xho* I), and 1x of enzyme buffer D (Promega). Digestion was carried out at 37°C for 1 h and the digestion products were then analysed by electrophoresis using 1.2% (w/v) agarose gel with 0.1 µg ml^{-1} EtBr in 1x TBE buffer. Gels were run at 70mA for about 1.5 h. and a 1 kb ladder was used as molecular marker to check the size of the fragments. The inserts where then purified from agarose gel and re-cloned in a pGEM-T easy Vector

DNA sequencing was carried out as described in Section 2.1.12.20.

The DNA sequences were analysed and compared with the GenEMBL databases, and the derived amino acid sequences were compared with the SWIIS-PROT databases, using the FASTA program or the BLAST network service (NCBI) in both cases.

GCG Package, version 7 and PRETTY-BOX programmes were used for PILEUP alignement.

CHAPTER 3

Physiological Studies of Source / Sink relationships in the 'Stay green' Mutants.

3.1. Introduction

Yield and percentage protein are key issues in the production and marketing of wheat. Yield is a product of the activity of processes contributing to deposition of starch in the grain, and protein percentage reflects processes of nitrogen metabolism. Starch accounts for most (approximately 80%) of the grain dry weight, making yield almost completely dependent on deposition of starch in the endosperm. Starch and protein accumulate in the endosperm from precursors (sucrose for starch synthesis and amino acids for protein synthesis) supplied by the rest of the plant. Limits to the rate of starch deposition involve a balance between the capacity of the plant to produce substrate (source limited) and the capacity of the grain to utilise it (sink limited). Furthermore, protein deposited in the grain is derived mainly from the protein in the leaf (ribulose 1,5-bisphosphate carboxylase oxygenase) that is directly responsible for fixing CO_2 to produce the substrate for starch deposition.

Therefore, altering the source/sink relationships in maturing crop plants may modified both yield and protein percentage.

The source/sink relationships in plants exhibiting the 'stay green' trait are not clear. Although a significant proportion of leaf protein N is immobilised in the old, non-photosynthetic leaves of type C 'stay green', there are no differences in the rates of dry matter production (Bakken *et al.*, 1997).

However, an extension of the grain filling period by extending the period of photosynthetic activity during senescence, may alter the rate and duration of both starch and protein deposition.

The aim of this chapter is understanding the relationships between leaf and seeds metabolism in a different class of 'stay green' mutants to type C.

3.2. Results

3.2.1. Isolation of durum wheat mutants with delayed leaf senescence

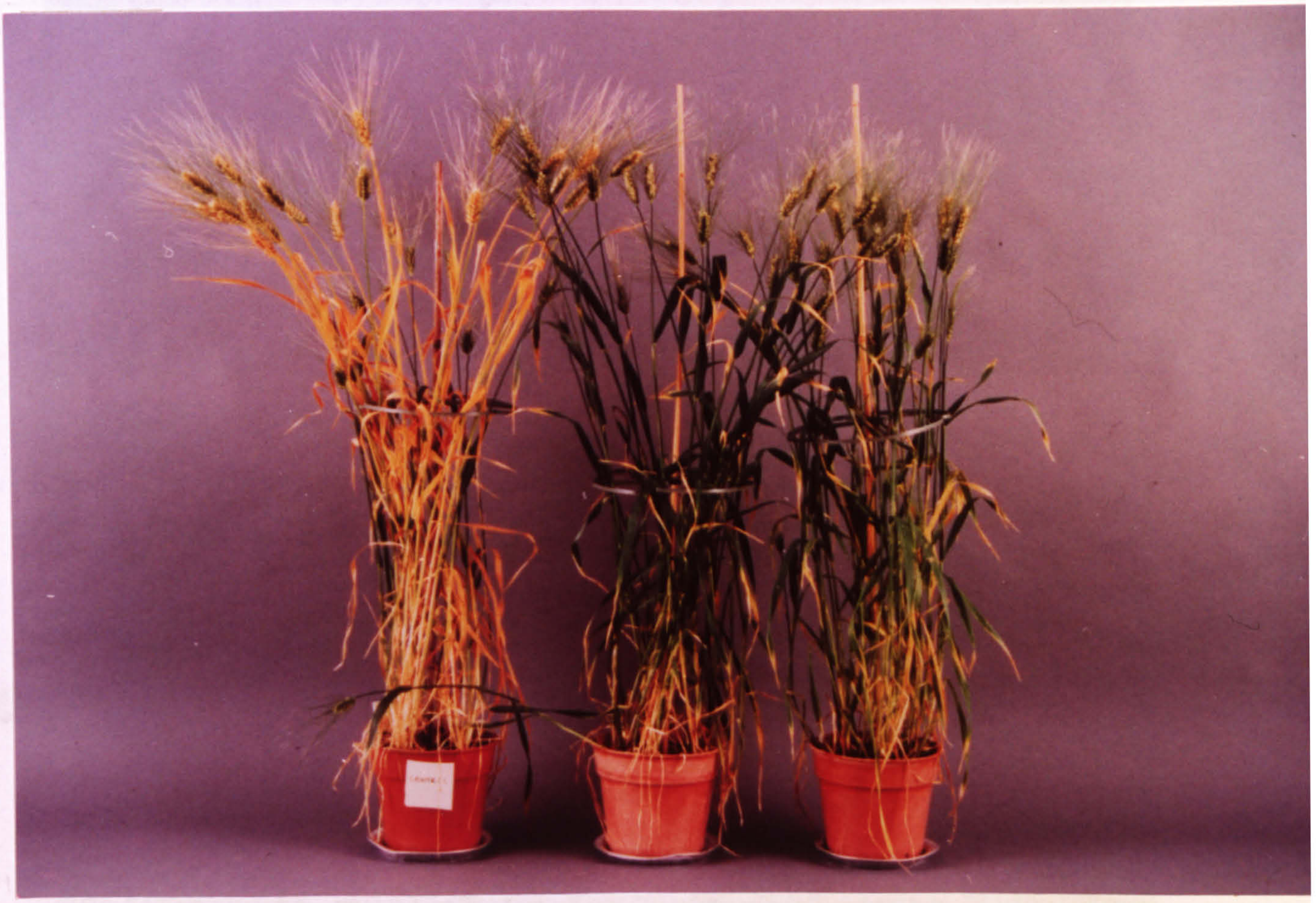
Mutant plants with delayed leaf senescence were identified by screening seed pools mutagenised either with EMS or with sodium azide. Initial screening was carried out in the field by visual evaluation of the degree of leaf yellowing resulting by chlorophyll loss, and four mutants were chosen for their similar timing of flowering but late timing of senescence.

All of these mutant lines were isolated from the EMS population and were named **139, 142, 196**, and **504**.

Delayed chlorophyll loss in the leaves of the mutant plants is evident compared with the parental phenotype (**Fig.3.**). Although the apparent delay of leaf senescence could result from delayed flowering time, the time of flowering, measured as the time of the appearance of the pollen, was only two days later in mutants **139** and **142** compared with the control plants while the flowering time of the mutants **504** and **196** was the same. However, even if a small difference did occur in the time of flowering, the leaf age (taking the flowering time as starting point) was equalized in all the experiments to avoid the effects of flowering differences on the mutant plants.

No differences were found in the numbers of leaves, either between mutant plants or between mutant and control plants. Furthermore, no significant differences were found in the number of seeds per ear (**Table 3.1.**).

Fig. 3. illustrates the senescence process in the 'stay green' mutants of durum wheat. In the control plants senescence progresses normally, with the flag leaves becoming senescent before the stem does. However, in the 'stay green' mutants this phenotype is inverted with the flag leaf remaining almost unchanged in colour, while the stem becames completely senescent. This particular phenotype may suggest that, in control plants, the sink is not a limiting factor, but that the source could be limiting. In the 'stay green' mutants, however, the sink capacity has, apparently, reached its maximun although a significant amount of nutrients can be translocated. Therefore, the source is not a limiting factor, but the sink could be limiting.



C mutant 139 mutant 504

Fig.3. 'Stay green' phenotype in durum wheat.

C: control plant

Fig. 3.1. The senescence process in the 'stay green' mutants of durum wheat.

In the control plants (**A**) senescence progresses normally, with the flag leaves becoming senescent before the stem. However, in the 'stay green' mutants this phenotype is inverted with the flag leaf remaining almost unchanged in colour, while the stem becames completely senescent (**B**).

Plants	Nr of leaves per plant (after booting)	Nr of seeds per ear (at harvesting)	Plants height (cm) (at maturity)
Control	5	46	85
Mutant 504	5	44	85
Mutant 196	5	46	85
Mutant 139	5	47	80
Mutant 142	5	46	80
LSD(p=0.05)		3.5	6.3

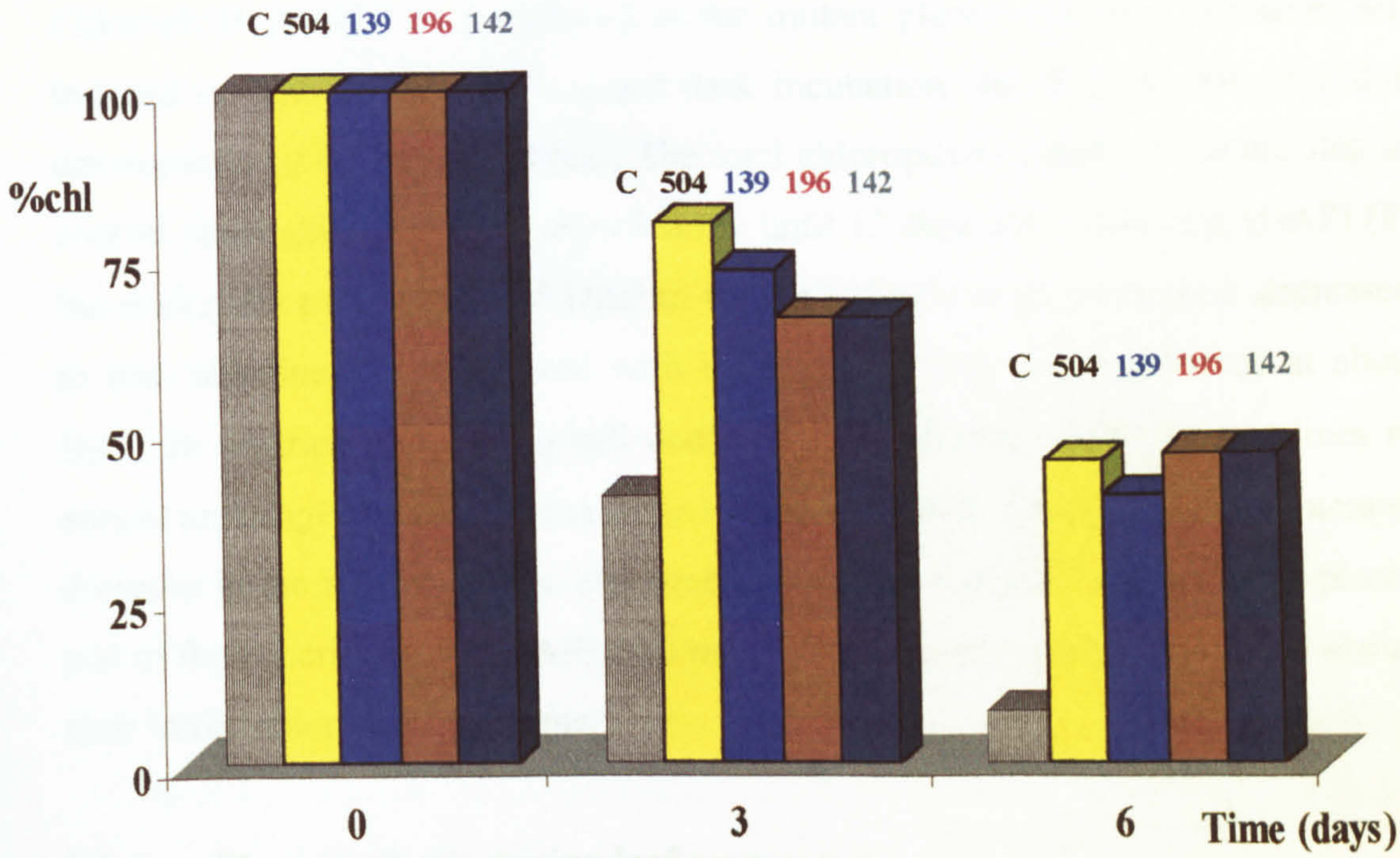
Tab. 3.1. Comparison of phenotypes of control and mutant plants

Plants were grown in 25 cm pots in a controlled greenhouse in Long Ashton Research Station (LARS, Long Ashton, Bristol, UK). Each value is the mean value of 15 replications. No significant differences were found in the number of seeds per ear.

3.2.2. *in vitro* experiments

Incubation of detached leaves in darkness (dark screening) has been widely used in studies of leaf senescence and was employed here to accelerate leaf senescence in a reproducible manner.

Senescence was induced by detaching the fifth youngest fully-expanded leaf of both parental and mutants plants and incubating in darkness. Total chlorophyll decreased in the leaves of parental and mutant lines during the course of dark-induced senescence (**Fig. 3.2.**). There were no significant differences at the beginning of the experiment (day 0). Although total chlorophyll decreased significantly between days 0 and 3 in all the mutants, the rate was significantly greater and statistically more strongly significant ($p=0.05$) for the control plants. Indeed, after 3 and 6 days of dark-induced senescence, the mutants were able to retain more than 50% and 40%, (139 and 142, 504 and 196 respectively), of their initial chlorophyll contents compared with the control leaves. However, no significant differences were found between the mutants.



	Chl a (µg cm^{-2})			Chl b (µg cm^{-2})			Total Chl (µg cm^{-2})		
	0d	3d	6d	0d	3d	6d	0d	3d	6d
Control	76,2	40,1	3,3	56,1	28,4	1,2	132,3	68.5	4.5
139	75,7	55,0	34,7	54,8	33,1	17,2	130,5	88,1	51,9
142	75,1	58,0	38,4	54,9	36,1	22,0	130,0	94,1	60,4
504	76,5	60,1	38,2	55,5	35,9	20,9	132,0	96.0	59,1
196	76,1	57,8	37,8	55,2	36,0	21,2	131,3	93,8	59,0

Fig. 3.2. Changes in the total chlorophyll contents of senescing leaves of control plants (cv Trinakria) and 'stay green' mutants incubated in continuous darkness for six days (0d → 6d).

Leaves were excised and placed in the dark to induce senescence. Control (stage 0) were freshly harvested, untreated leaves. Total chlorophyll content is expressed either as percentuage of mature green leaf chlorophyll levels or as µg cm^{-2}. **C**= Control
LSD (p=0.05) = 0.236.

3.2.3. Chlorophyll contents during leaf senescence

Chlorophyll stability is manifested in the mutant plants, not only in tissues artificially induced to senesce by excision and dark incubation (see Fig.18), but also during the development of an individual leaf. The total chlorophyll content of mature flag leaves of control plants did not change significantly until 15 days after flowering (DAF) (**Fig. 3.3.**) but during the period from 15 DAF to 40 DAF the chlorophyll content decreased falling to zero and this was associated with visible yellowing being observed at about 20-25 DAF. In contrast, the chlorophyll contents of the leaves of the mutant lines remained almost unchanged over the same period. However, after the chlorophyll content started to decrease in the mutant plants, it proceeded as a similar rate to the control plants. At the end of the experiment (40 DAF), two of the four mutants analysed retained about 50% of their initial chlorophyll contents.

3.2.4. Photosynthesis during leaf senescence

Changes in the photosynthetic apparatus were investigated during maturation and senescence of the flag leaves of both mutant and control plants. Determination of net photosynthesis (Pn) and efficiency of photosystem II, were carried out at weekly intervals from flowering until full senescence of plants. Furthermore, parameters such as leaf temperature, chamber [CO_2] and light intensity, which could affect the photosynthetic process, were monitored and were observed to be similar throughout the experiments **(Table 3.2.)**.

The patterns of chlorophyll breakdown was the same as observed for Pn. Photosynthesis in the control plants was highest at about 10 - 15 DAF and after this date decreased steadily until it reached zero (at 40 DAF). In contrast, the decline of photosynthesis in the mutants started at least one week later than in the control plants (**Fig. 3.4.**). At the end of the experiment (40 DAF), two of the four mutants analysed were able to retain about 50% of their initial rate of photosynthesis. Therefore, in all the mutants delayed chlorophyll breakdown was followed by an extension of photosynthesis. This is shown, particularly, in **Fig. 3.5.** which plots the relationship between Pn and chlorophyll content for leaves of all ages. The curves show an association between chlorophyll and Pn, with low levels of

chlorophyll in older leaves being associated with low Pn in both control and mutants plants. However, a marked delayed of net photosynthesis and chlorophyll content can be observed in all the four mutants analysed. One interesting feature was that both control and mutant plants showed early decreases in photosynthesis before decreases in chlorophyll content was observed. Indeed, the decline in net photosynthesis started at about 13 and 20 days after flowering in control and mutant plants, respectively, but chlorophyll breakdown started about 17 and 25 days after flowering in control and mutant plants, respectively.

These result suggest that net photosynthesis and chlorophyll content are not necessarily coupled during senescence in durum wheat and may be controlled by independent regulatory mechanisms. Evidence that the greater retention of chlorophyll by the mutants was associated with comparable maintenance of Pn was also provided by sub-stomatal CO_2 (Ci) measurements. Indeed, the rate of increase of Ci from flowering until full senescence, was lower for all the mutants analysed than for control plants (**Fig. 3.6.**).

3.2.5. PS II efficiency during leaf senescence

Most *in vitro* studies, using isolated thylakoid membranes of chloroplasts from senescing leaves, have showed that senescence results in a decrease in the photochemical activities of PS I and PS II (Grover and Mohanty, 1992). However, PS II is more susceptible to senescence than PS I and a greater decrease in PS II activity is observed during senescence of leaves (Grover *et al.*, 1986). Therefore, in addition of net photosynthesis, the efficiency of photosystem II was determined as F_v/F_m (Genty et al., 1989). Maximun values of about 0.6 were obtained for both mutants and control plants for fully developed leaves (**Fig. 3.7.**), however, as for net photosynthesis, the F_v/F_m ratio decrease steadily until it reached zero (40 DAF) in the control plants.

In contrast, in all the mutants examined, only minor variation was observed in the decline of the efficiency of photosystem II until 25 DAF. Therefore, the photosynthesis was the extended in the leaves of mutants plants, with maintained high efficiencies of PS II.

However, after the F_v/F_m ratio started to decrease in the mutant plants the net photosynthesis proceeded as in the control plants.

The F_v/F_m value is not dependent on the number of chloroplast or the amount of chlorophyll but only on the efficiency of light capture (John *et al.*, 1995), therefore, the delayed decrease in the F_v/F_m value during senescence of the mutants leaves being an additional indication that functional photosynthetic units are maintained longer in the mutants than in the control plants.

Thomas and Smart (1993) classified 'stay green' lines of crop plants according to the degree to which pigment stability is reflected in photosynthetic capacity. Figures 3.3. and 3.4., therefore, confirm that for net photosynthesis, the 'stay green' mutants in durum wheat have the photosynthetic characteristics of functional *'stay green'* phenotype.

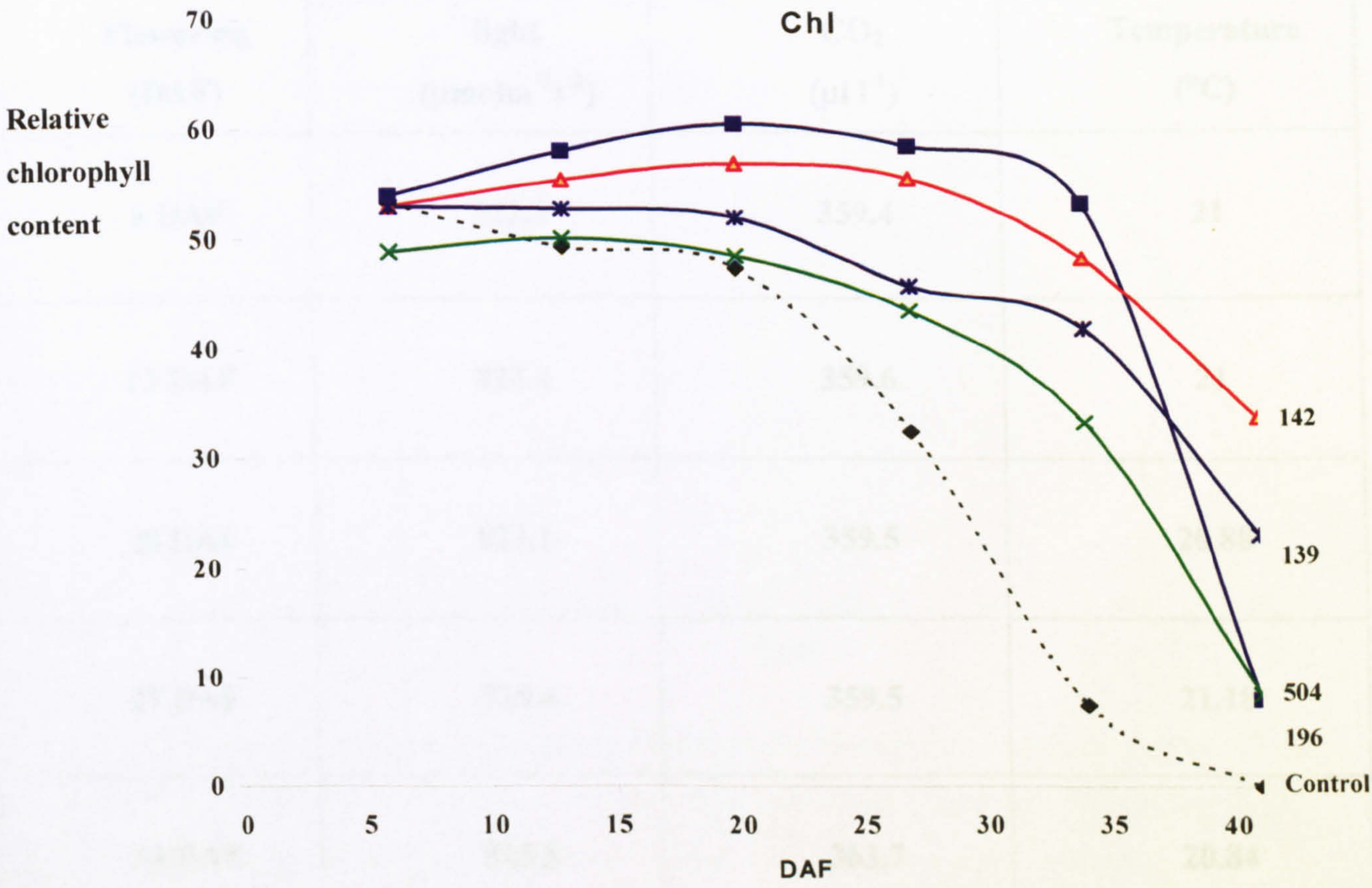


Fig. 3.3. Differences in relative chlorophyll contents between mutants and control plants

Readings were taken on the flag leaves of eight individual plants from flowering until full senescence. Each of the six numbered values is the mean of eight replicates.

LSD ($p = 0.05$) **a** = 5.25 for comparing between plants; **b** = 5.04 for comparing between times within a single plant.

Days After Flowering (DAF)	Chamber light ($\mu molm^{-2}s^{-1}$)	Chamber CO_2 ($\mu l\ l^{-1}$)	Leaf Temperature (°C)
6 DAF	822.3	359.4	21
13 DAF	822.4	359.6	21
20 DAF	822.1	359.5	20.88
27 DAF	825.4	359.5	21.18
34 DAF	825.5	363.7	20.84
LSD (p=0.05)	3.4	2	0.32

Tab 3.2. Measurement of chamber [CO_2], leaf temperature and light were taken for each plant (eight replication in total) during analysis of 'stay green' mutants and control plants of *Triticum durum*.

The above parameters showed no evidence of significant differences between plant types nor any interactions between plant types and time was found. Each of the six numbered values is the mean of eight replicates.

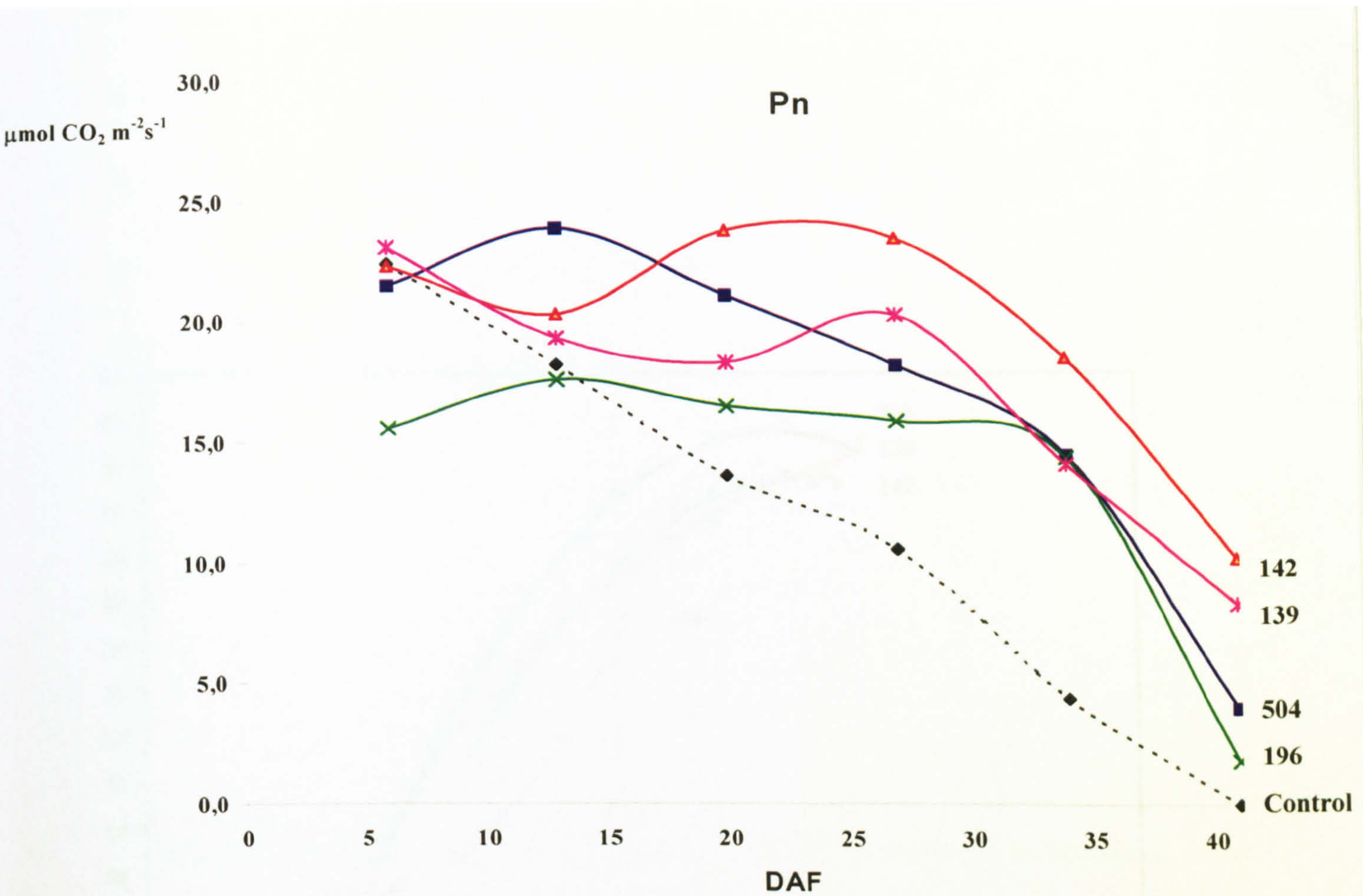


Fig.3.4. Differences in net photosynthesis between mutants and control plants

Readings were taken on the flag leaves of eight individual plants from flowering until full senescence. Each of the six numbered values is the mean of eight replicates.

LSD (p= 0.05) **a** = 3.85 for comparing between plants; **b** = 3.01 for comparing between times within a single plant.

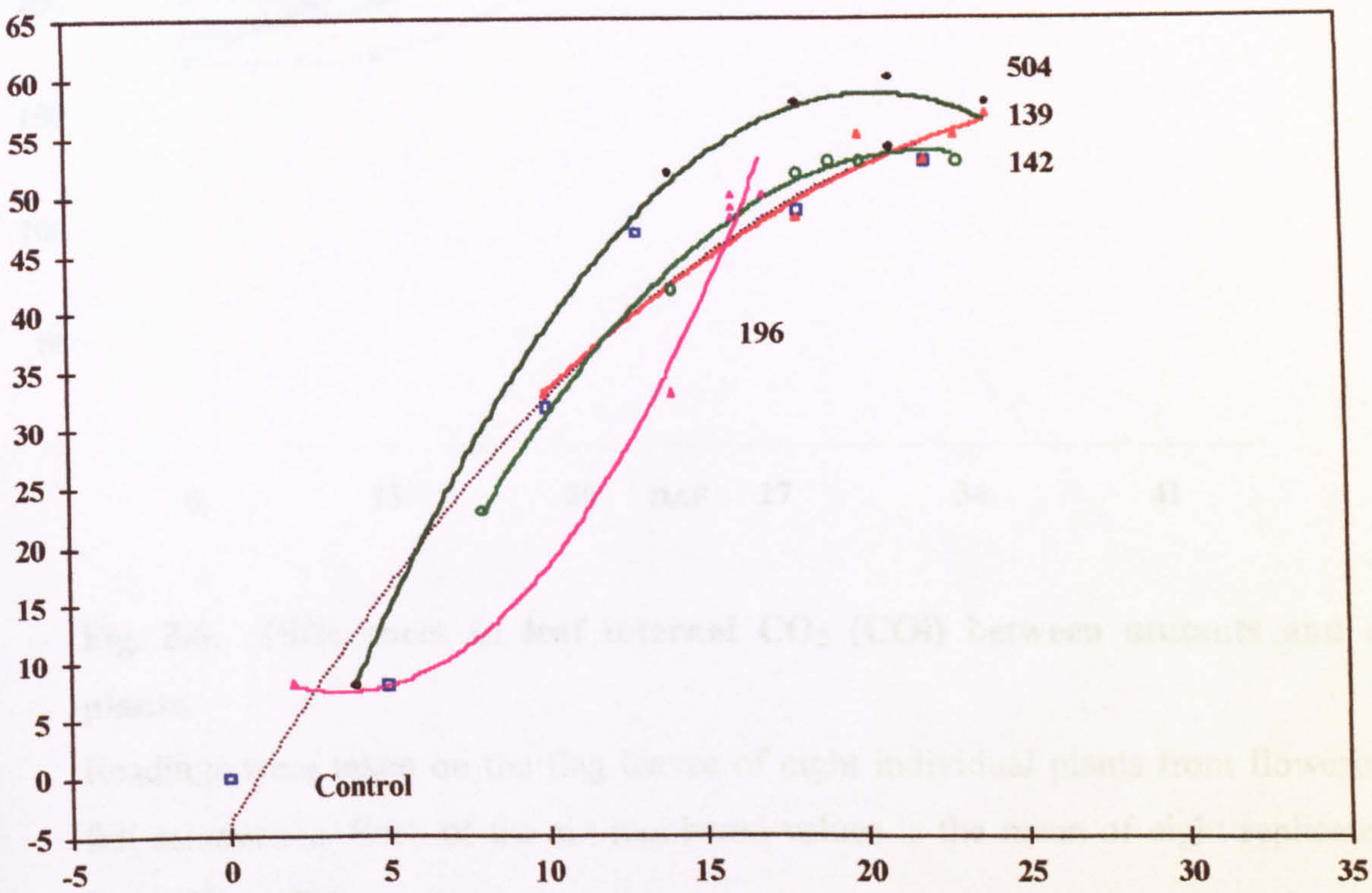


Fig. 3.5. Relationship of net photosynthesis with chlorophyll from flowering until full senescence of mutant and control plants.

Readings were taken on the flag leaf of eight individual plants. Each of the six numbered values is the mean of eight replicates.

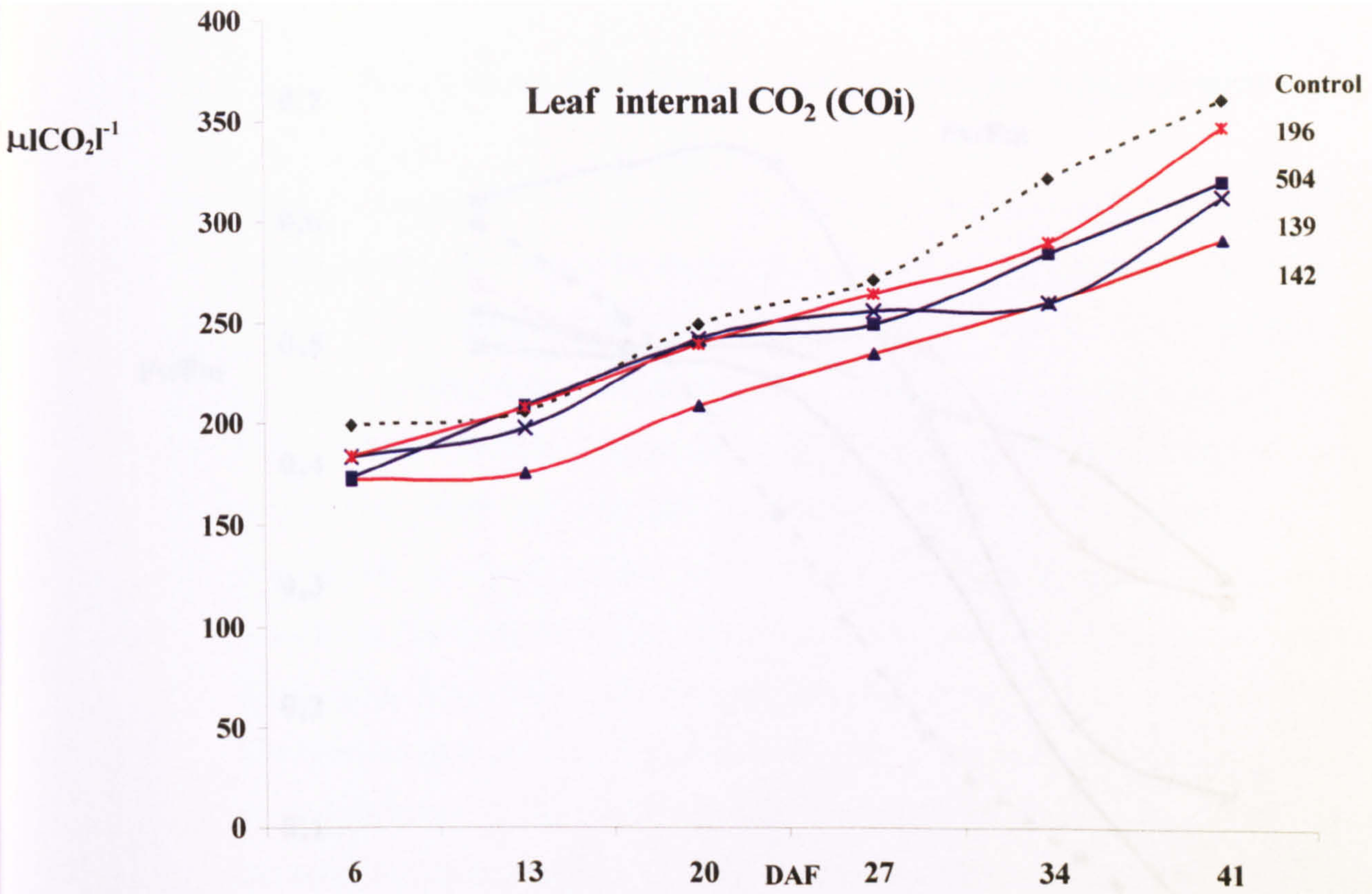


Fig. 3.6. Differences in leaf internal CO_2 (COi) between mutants and control plants.

Readings were taken on the flag leaves of eight individual plants from flowering until full senescence. Each of the six numbered values is the mean of eight replicates. LSD ($p= 0.05$) = 18.3.

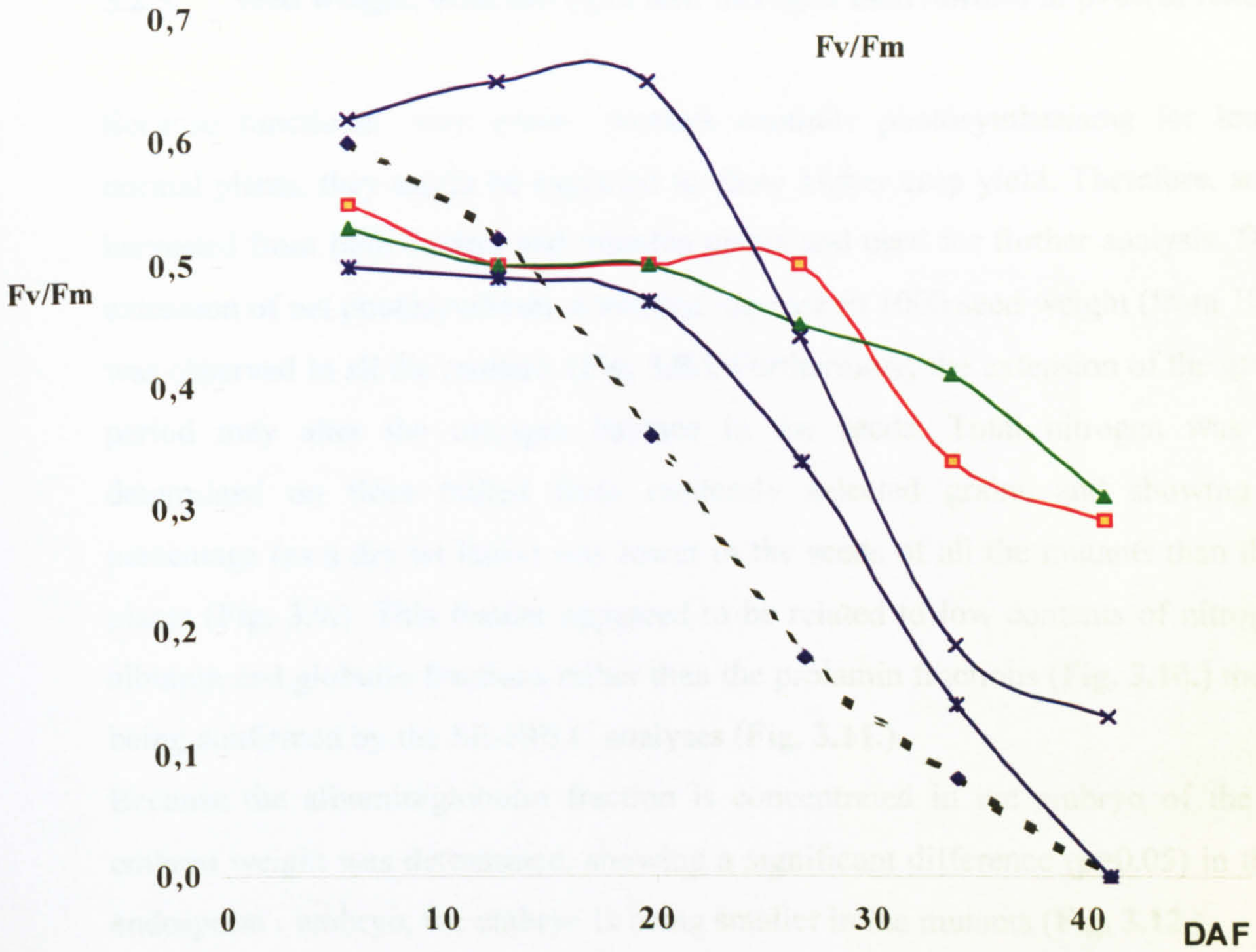


Fig. 3.7. Differences in efficiency of PS II (F_v/F_m) between mutants and control plants. Readings were taken on the flag leaves of eight individual plants from flowering until full senescence. Each of the six numbered values is the mean of eight replicates. LSD (p= 0.05) **a** = 0.11 for comparing between plants; **b** = 0.14 for comparing between times within a single plant.

3.2.5. Seed weight, total nitrogen and nitrogen distribution in protein fractions

Because functional *'stay green'* mutants continue photosynthesising for longer than normal plants, they might be expected to show higher crop yield. Therefore, seeds were harvested from both control and mutants plants and used for further analysis. Due to the extension of net photosynthesis, a marked increase in 1000 seed weight (from 10 to 14%) was observed in all the mutants (**Fig. 3.8.**). Furthermore, the extension of the grain filling period may alter the nitrogen balance in the seeds. Total nitrogen was therefore determined on flour milled from randomly selected grains and showing that the percentage (as a dry wt basis) was lower in the seeds of all the mutants than the control plants (**Fig. 3.9.**). This feature appeared to be related to low contents of nitrogen in the albumin and globulin fractions rather than the prolamin fractions (**Fig. 3.10.**) these results being confirmed by the SE-HPLC analyses (**Fig. 3.11.**).

Because the albumin/globulin fraction is concentrated in the embryo of the seed, the embryo weight was determined, showing a significant difference ($p=0.05$) in the ratio of endosperm **:** embryo, the embryo is being smaller in the mutants (**Fig. 3.12.**).

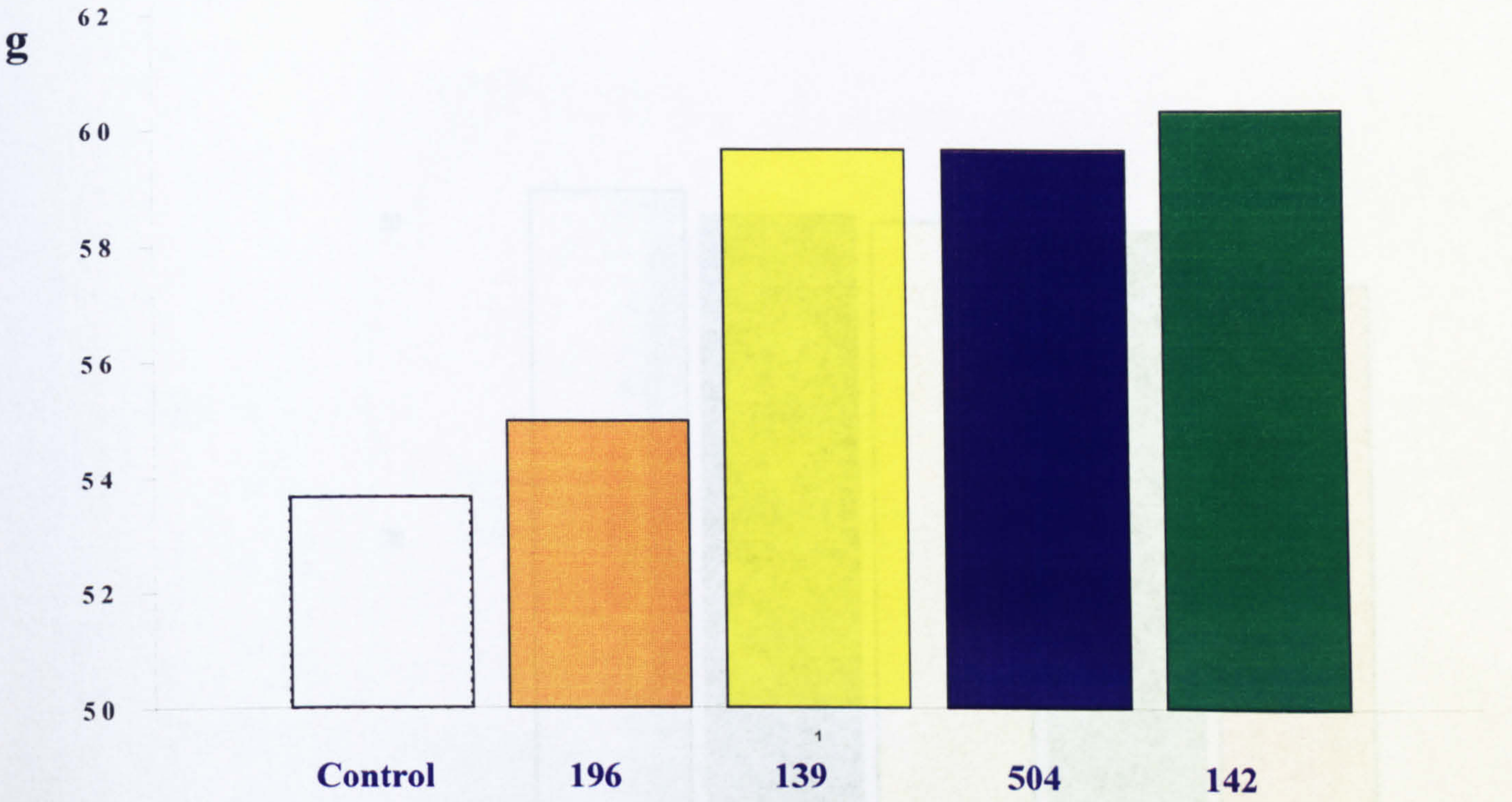


Fig.3.8. 1000 seed weights of mutants and control plants.

Seeds were harvested, counted and the dry seed weights was calculated.

Each value is the mean of three replicates. LSD(p=0.05) = 0.8771

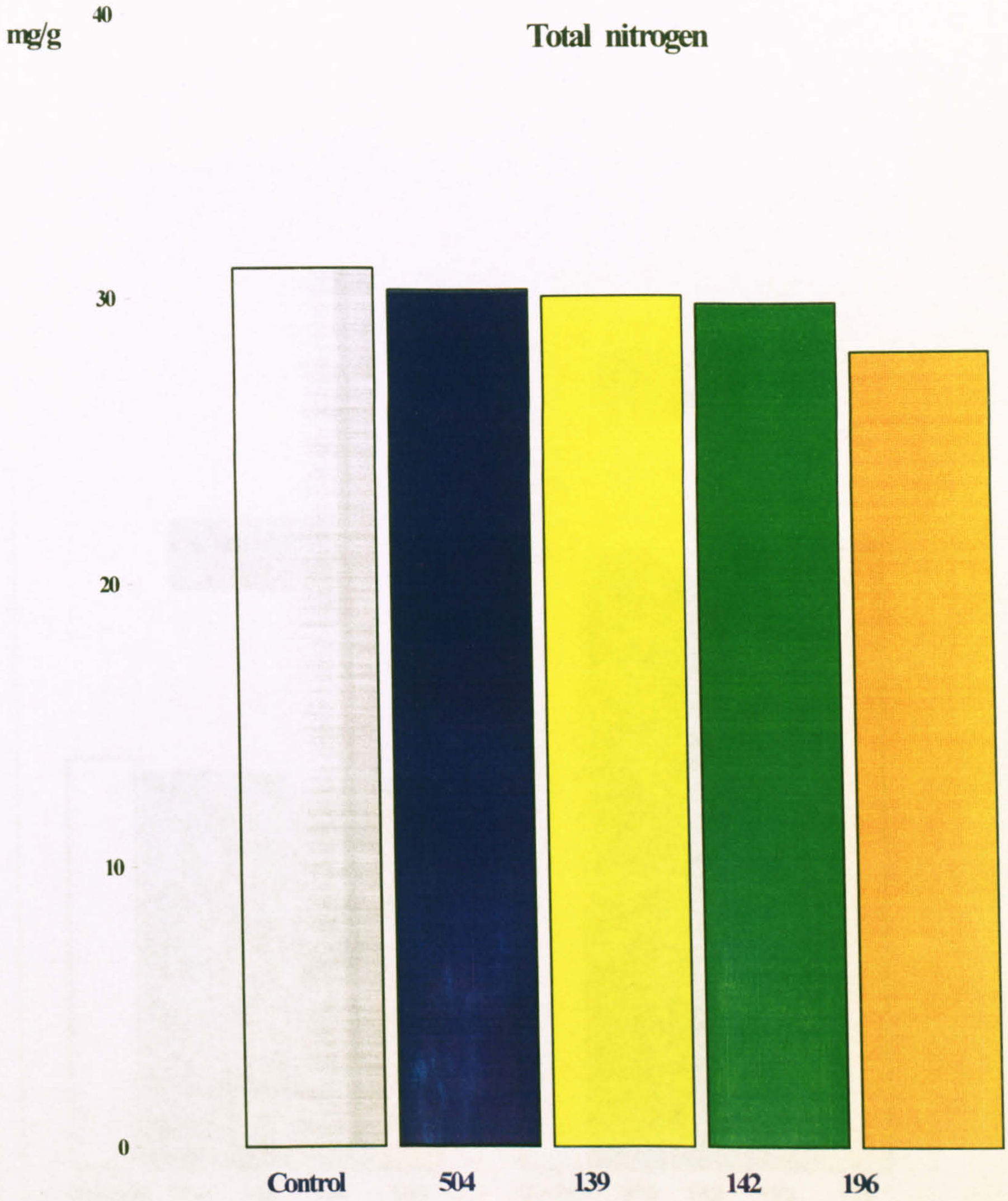


Fig.3.9. Total nitrogen contents in the seeds of mutants and control plants.

Each value is the mean of five replicates.

LSD (p=0.05) = 0.17

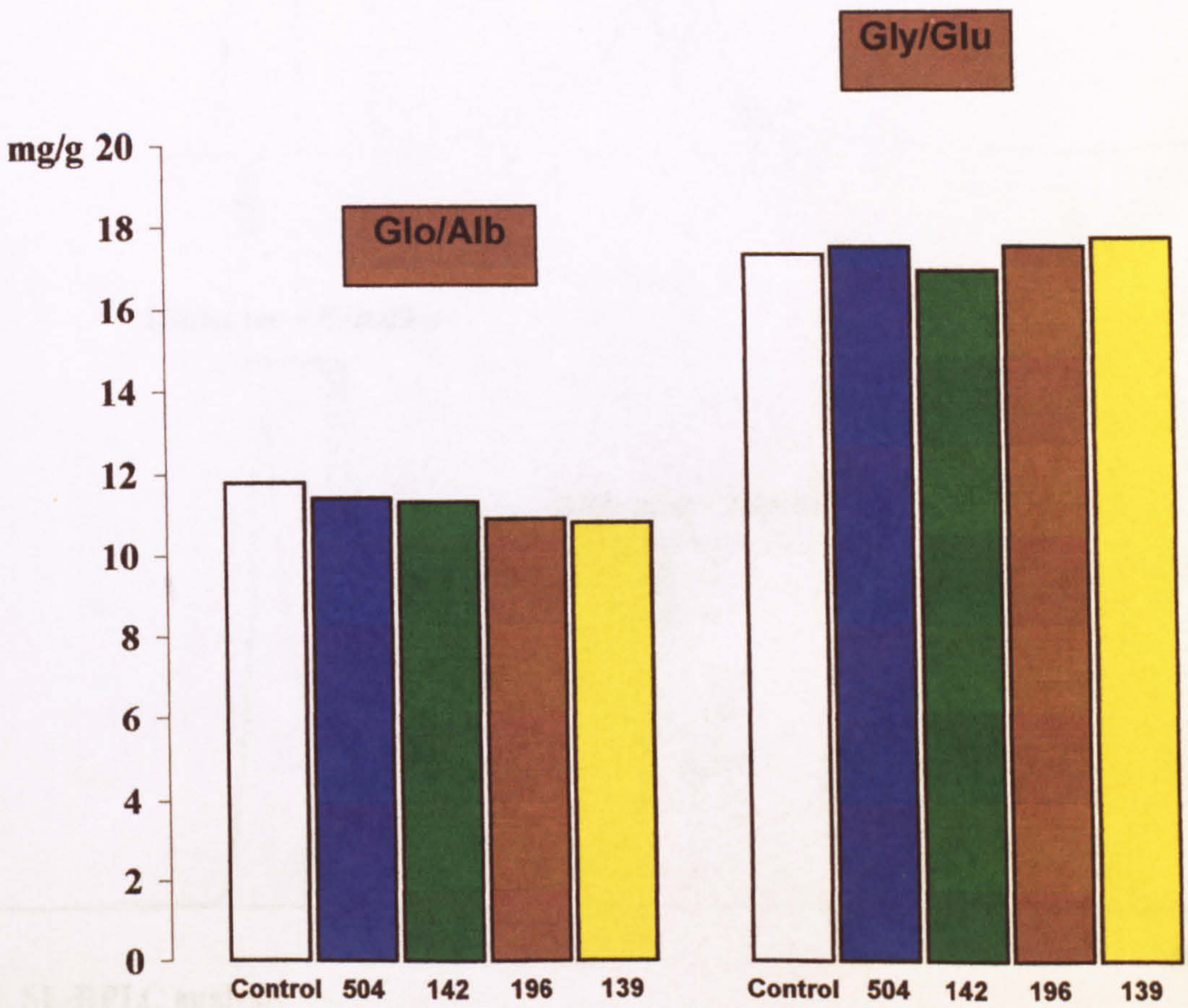


Fig. 3.10. Nitrogen partition in storage proteins.

LSD (p=0.0.5) **a** = 0.12 for comparing between the globulins and albumins (Glo/Alb) fraction and **b** = 0.21 for comparing between the gliadins and glutenins (Gly/Glu) fraction.

Each value is the mean of five replication.

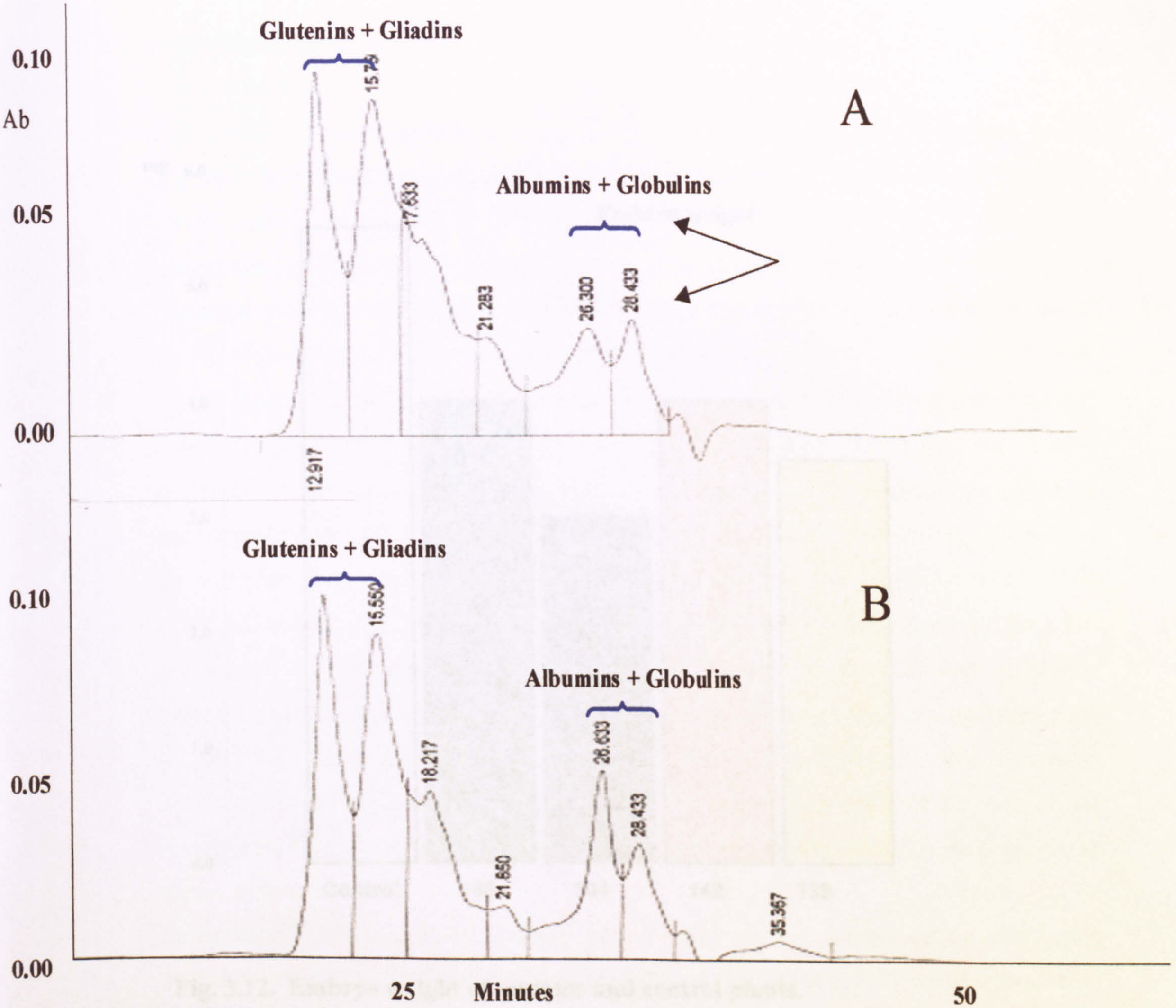
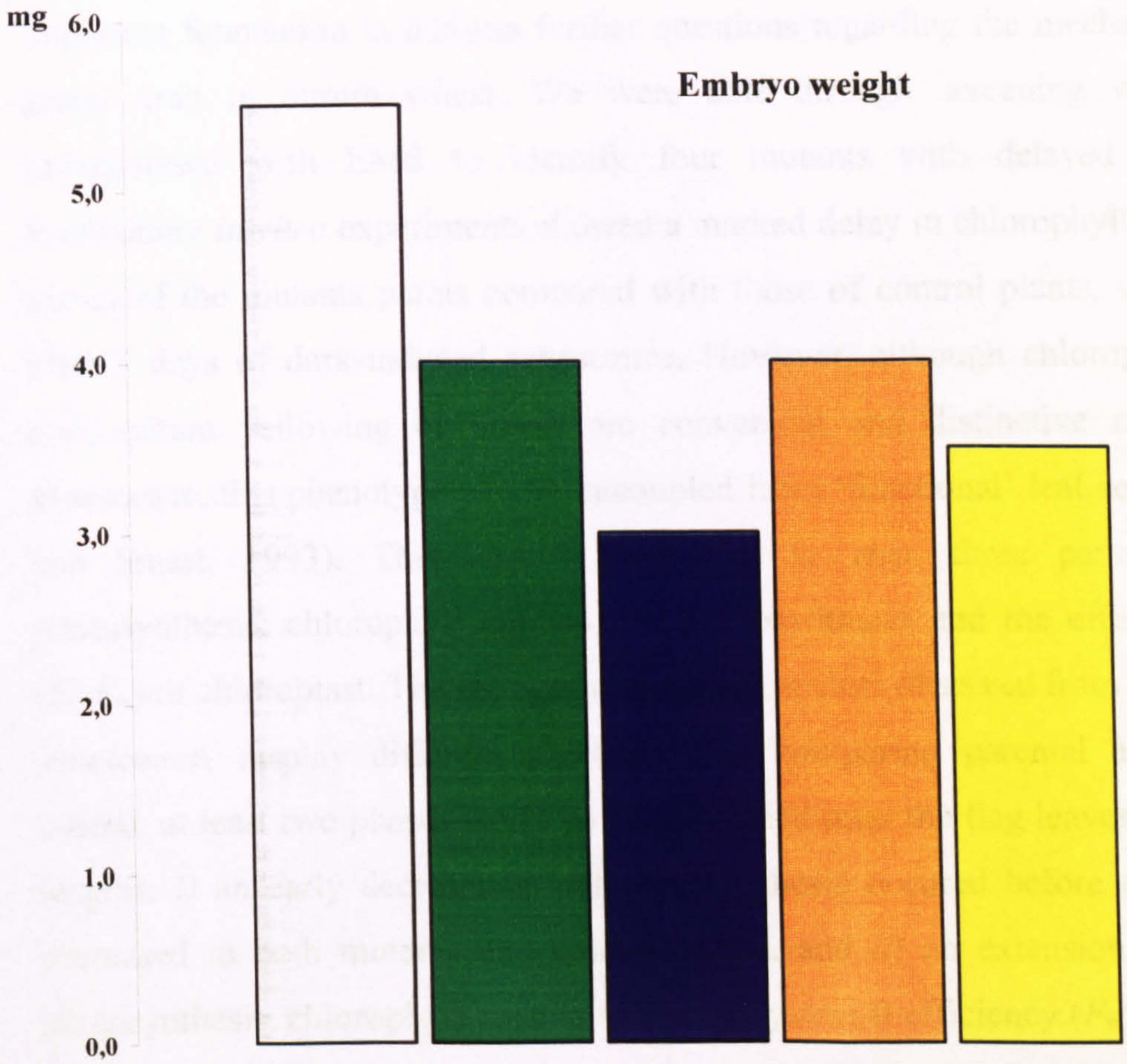


Fig.3.11. SE-HPLC analysis

SE-HPLC analysis on flour milled of randomly picked grain. Arrows show the main differences between control (B) and mutant 139 (A).



Control	196	504	142	139

Fig. 3.12. Embryo weight of mutants and control plants.

Each value is the mean of five replicates.

LSD (p=0.0.5) = 0.8

3.2.7. Discussion

The identification of mutants that are defective in aspects of leaf senescence is an important foundation to address further questions regarding the mechanism of the 'stay green' trait in durum wheat. We were able through screening with a population mutagenased with EMS to identify four mutants with delayed leaf senescence. Preliminary *in vitro* experiments showed a marked delay in chlorophyll breakdown in the leaves of the mutants plants compared with those of control plants, which was evident after 3 days of dark-induced senescence. However, although chlorophyll loss and the concomitant yellowing of leaves are convenient and distinctive indications of leaf senescence, this phenotype can be uncoupled from 'functional' leaf senescence (Thomas and Smart, 1993). Therefore, I measured, *in vivo,* three parameters related to photosynthesis: chlorophyll content, net photosynthesis and the efficiency of the PSII (F_v/F_m) of chloroplast. The changes in these parameters observed from flowering until full senescence, display different kinetics when comparing parental and control plants. Indeed, at least two phases could be distinguished after the flag leaves reached their final lengths: *i)* an early decrease in net photosynthesis occured before chlorophyll content decreased in both mutants and control plants, and *ii)* an extension of the rate of net photosynthesis, chlorophyll content and photosystem II efficiency (F_v/F_m) were evident in all the mutants analysed.

Differences in the kinetics of decline of chlorophyll content and photosynthesis during senescence have also been reported by Friedrich and Huffacker (1980) and Humbeck *et al* (1996). The marked early decrease in net photosynthesis may indicate that photosynthesis and chlorophyll content are not necessarily coupled during the senescence of durum wheat but may be controlled by independent regulatory mechanisms. The drastic decrease in the chlorophyll content of the flag leaves indicates that the senescence processes started at least one week earlier in the control than in the mutants plants. The maximum efficiency of PS II photochemistry measured as F_v/F_m, showed no significant changes until the onset of senescence in mutants plants (about 25 DAF). Furthermore, the extended photosynthesis and chlorophyll contents in the leaves of mutant plants was accompained by an high efficiency of PS II photochemistry. However, after the onset of

senescence, the decreases in all the parameters analysed proceeded as in the control plants.

Because all the photosynthetic parameters seem to be affected in the mutants, we conclude that the novel mutants have the characteristics of functional '*stay green*' according to Thomas and Smart (1993).

This type of 'stay green' mutant may arise by alteration of genes involved in the timing of the initiation of senescence or in the regulation of its rate of progress (Smart, 1994).

The source/sink relationships in plants exhibiting the 'stay green' trait are not clear.Starch and protein are synthesised in the endosperm from precursors (sucrose for starch synthesis and amino acids for protein synthesis) supplied by the rest of the plant. Limits to the rate of starch deposition involve a balance between the capacity of the plant to produce substrate (source limited) and the capacity of the grain to utilise it (sink limited). Therefore, altering source/sink relationships in maturing crop plants may modify both yield and protein percentage. Furthermore, an extension of the grain filling period may alter the rate and duration of both starch and protein deposition.

Since the 'stay green' phenotype in durum wheat continues to photosynthesise for longer than normal, it might be expect to result in higher yield of crops. Indeed, a significant increase in seed weight (by about 10% to 12%) was observed in all the mutants analysed supporting the hypothesis that the extension of grain filling may increase the yield of durum wheat.

Furthermore, several major effects on sink physiology were observed related to the extension of photosynthesis :

1. An increase of seed weight was observed in all the mutants, demonstrating that that the 'stay green' trait could be of value for increasing the yield of crops (Thomas and Smart, 1993).

2. An increase in photoassimilate translocated to the sink is expected, which could result in increased starch synthesis and hence increased seed weight in the mutants.

3. The protein percentage (often referred as protein content) is a ratio value and it is dependent on the amounts of both protein and starch (Jenner *et al*, 1991). Consequently an increase in the starch content of the seeds could explain a decrease in total nitrogen observed in all the mutants.

4. Futhermore, less nitrogen was present in the albumin/globulin fraction. This fraction, mainly constituted by enzymes, is concentrated in the embryo which was smaller in the mutant.

CHAPTER 4

Comparative analysis between 'stay green' mutants and control plants

4.1. Introduction

Several techniques are available to detect variation in gene expression between populations of cells. These include subtractive hybridisation (SH), differential colony hybridisation (DCH) and mRNA differential display, all based on the analysis of mRNA. The first two techniques, however, are mainly qualitative methods which do not allow quantitative changes to be determined. In addition, large amounts of mRNA are required for SH and several rounds of screening for DCH. Differential display, a method reported by Liang and Pardee (1992) overcomes both of these limitations. It is based on the assumption that every cell expresses some 15,000 genes and, in principle, every individual mRNA molecule can be reverse transcribed and amplified, using a set of arbitrary primers, by the polymerase chain reaction. Theoretically, it is a powerful and a sensitive tool for determining altered pathways of gene expression between cellular populations. However, the high percentage of false positives generated by differential display (as high as 85%), has limited the potential of the method. Therefore, in order to simplify the procedure it is important to identify and to discard false positives prior to cloning.

4.2. Results

4.2.1. Differential display and selection of true differentially regulated bands prior to cloning

In order to isolate genes whose expression is up or down regulated by the 'stay green' mutants, a comparative analysis between mutants and control plants was performed by differential display of mRNA.

The type of 'stay green' behaviour previously described in Chapter 3 may result after alteration of genes affecting either the timing of the initiation of senescence or its rate of progress. Furthermore, because a decrease in net photosynthesis was observed before the chlorophyll content decreases in both control and mutant plants, chlorophyll concentration was used as indicator of the possible onset of senescence when selecting material for molecular analysis.

The starting material for total RNA extraction was the flag leaves of plants growing in a controlled glasshouse. On the base of the chlorophyll concentration the stage named F1 (**Fig.4.**) was chosen for further analysis. 2µl of mRNA (1mg/µl) from control plants and a mix of mRNA from all the four mutants (equal amount of each, 2µl as total mRNA) was used as template for first strand cDNA synthesis. For DDRT-PCR 160 primer combinations [4 $(dT)_{12}$ M primers and 40 5'-arbitrary primers] were used. To reduce the number of false positive that arise from differential display, two approaches were adopted:

1. Two cDNA preparations were made from each mRNA preparation.

   PCR was performed in parallel on these two preparations and differentially expressed bands were only accepted as positives if they appeared in both reactions (**Fig.4.1.**). Four anchored oligo(dT) primers were used in combination with 40 arbitrary 10-mer primers,

with either 60% or 70% GC content. However, no influence of the GC content on the number of bands was observed.

After initial inspection of the gels, ~ 70 bands were identified as being differentially expressed. 50 bands were present at higher levels in the mutants plants while 20 fragments were amplified to higher levels in control plants. The senescence process had already started [chlorophyll concentration is the 80% of the initial content (see Fig.29)] in the samples of control plants taken at the F1 stage. Therefore, the fragments differentially expressed in the control plants could represented genes expressed during leaf senescence.
All the bands were excised and the DNA extracted gel and reamplified.
66 of the 70 bands were successfully re-amplified and the differentially expressed fragments were then screened by reverse northern blotting (Zegzouti *et al.*, 1997).

2. Reverse northern blotting and cloning

Aliquots of the re-amplified fragments were quantified and the same amount of each fragment was used. The fragments were then run in duplicate on the same 1.4% agarose gel and the gel was blotted onto the nylon membrane to allow identical transfer of the RNA samples. The membrane was then cut and the duplicates membranes were hybridised, either to a total cDNA preparation from leaves of control plants, or to a total cDNA preparation from leaves of mutant plants (**Fig.4.2.**).
10 fragments were eliminated as false positive because of their similar patterns of expression in both mutant and control RNA. Furthermore, 8 clones failed to hybridise to the cDNA probes in either of the duplicates. These bands were considered to result from contaminating genomic DNA and therefore discarded.
The differentially expressed cDNA fragments were re-amplified, cloned and used for northern blot analyses.

Since bands excised from the polyacrylamide gel may contain several cDNA sequences (Bauer *et al.*, 1993), four recombinant colonies from each differentially expressed fragment were picked at random and partially sequenced.

Consequently, 56 different kinds of clones were obtained from the original 48 clones with sizes ranging from 0.15-0.4 kb.

Once the clones had satisfied all of the above criteria, their expression pattern was characterised.

A brief outline of the procedure is given in **Fig.4.3**.

4.2.2. Northern blot analysis and sequencing of the differential expressed fragments

In order to to confirm that a cDNA band isolated from a differential display gel was a true positive, the fragments were used as probes for hybridisation of northern blots carrying 10 μg total RNA from leaves collected at the same stages used for DDRT- PCR analysis.

Of the 56 clones amplified, only 50 fragments represent transcripts that were differentially expressed in leaves of the mutant plants. Of which, 40 clones (named **W1,.....W40**) represent transcripts with higher expression in leaves of mutants plants **(Fig. 4.4.a clones W9)**, while 10 clones (named **SGW1,.....SGW10**) represent transcripts with higher expression in leaf of control plants **(Fig. 4.4.b clones SGW1 and SWG2)**.

6 clones failed to hybridise to total RNA from both mutants and control plants. However, given the limited sensitivity of the northern blot, we considered that, among these bands, some may have corresponded to weakly expressed genes. Therefore, the 6 clones that gave no signal were retained for subsequent experiments.

32 clones were sequenced and the result of the homology search is presented in **Table 4.1**.

A partial sequence of one cDNA obtained by the DDRT-PCR technologies is shown in **Fig. 4.5**.

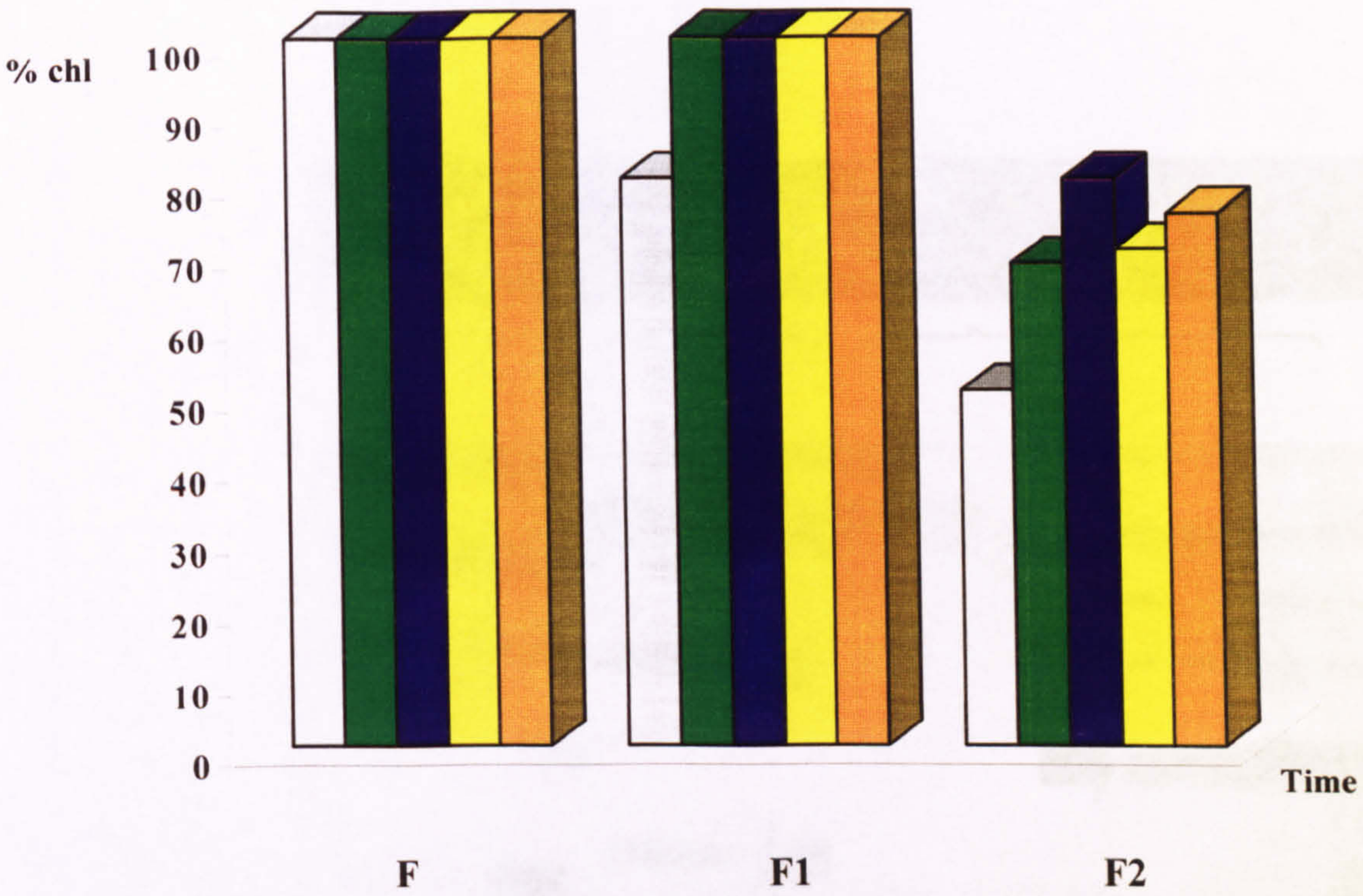


Fig. 4. Chlorophyll concentration from flower until full senescence. Plants were grown in a controlled glasshouse in LARS. Furthermore, on the base of the chlorophyll concentration the stage named F1 was chosen for further analysis. The chlorophyll content is expressed as percentage of the initial content. Three independent leaf were used for each chlorophyll extraction.

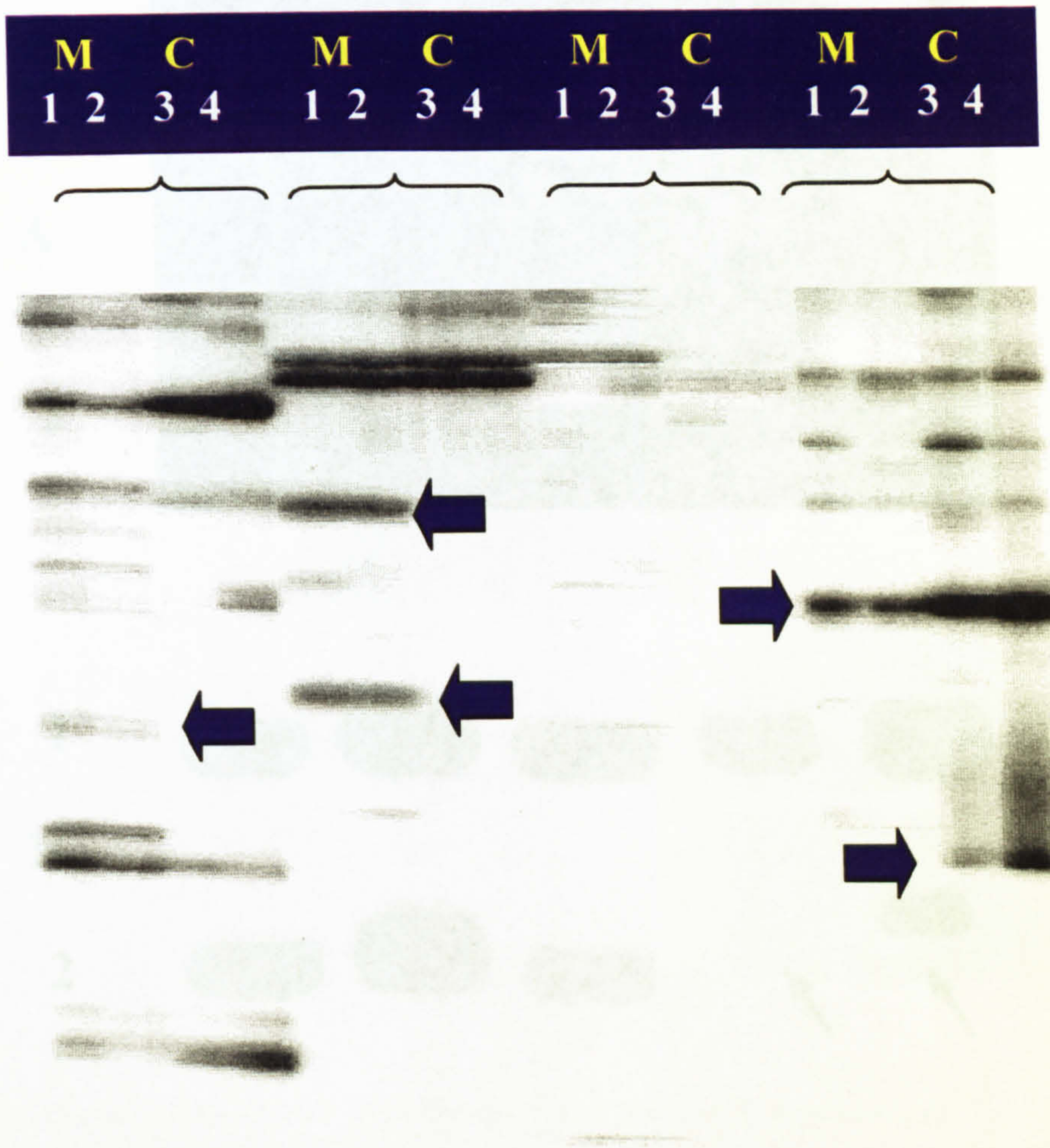
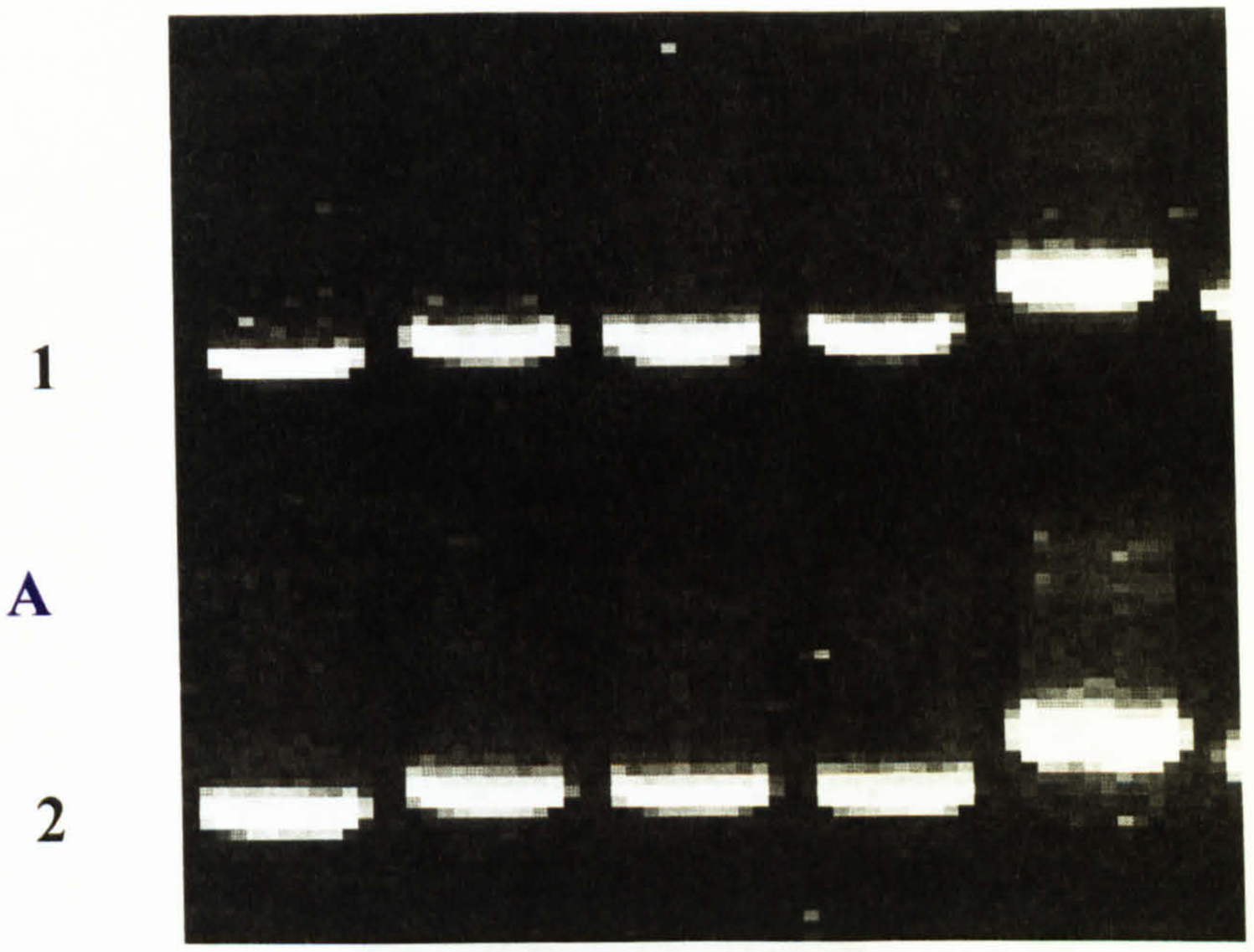


Fig. 4.1. A method to reduce the number of false positve.

Two cDNA preparations were made from each mRNA preparation. PCR was performed in parallel on these two preparations and differentially expressed bands were only accepted as positives if they appeared in both reactions.

M= mutant; **C**= Control



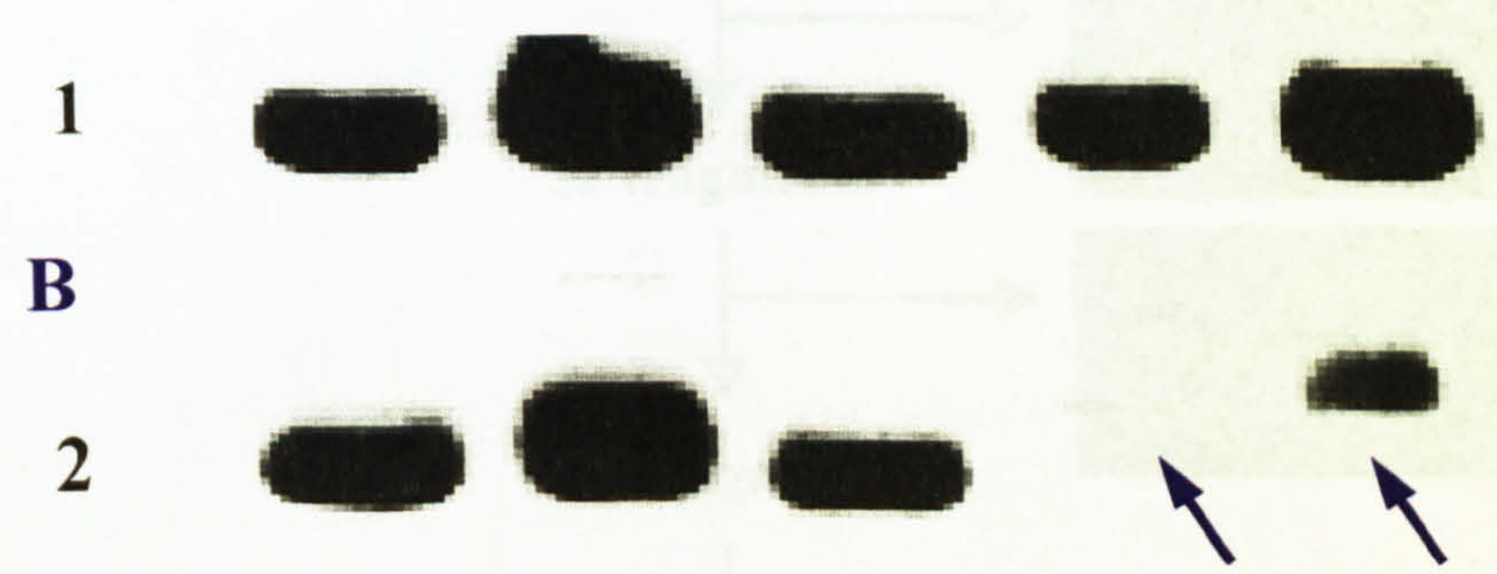


Fig.4.2. Elimination of false positive bands from DDRT-PCR analysis prior to cloning.

A) Differential bands eluted from the polyacrylamide gel were re-amplified and equal amount of the re-amplifed fragments were run in duplicate (**1** and **2**) on the same agarose gel.

B) Reverse Northern screening. The cDNA fragments were transferred onto a nylon membrane and the duplicate membrane were hybridised one to a total cDNA probe from senescing leaves of control plants (**1**) and the other to a total cDNA probe from senescing leaves of mutant plants (**2**). The arrows in Fig. 4.B indicate differential expressed bands.

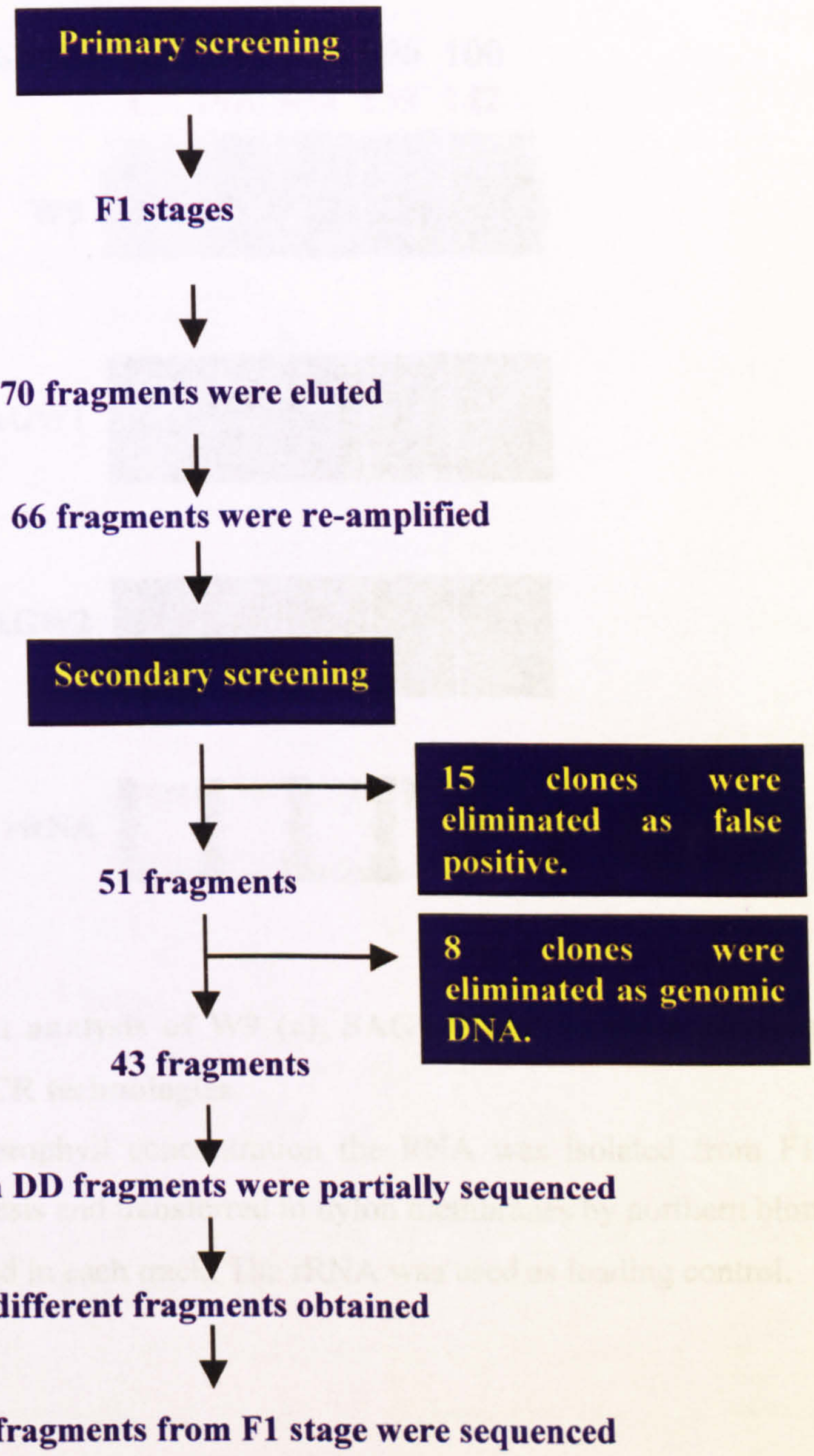


Fig. 4.3. An outline of DDRT-PCR procedure and results

The methodology adopted to discarded false positive is described in **Section 4.2.**

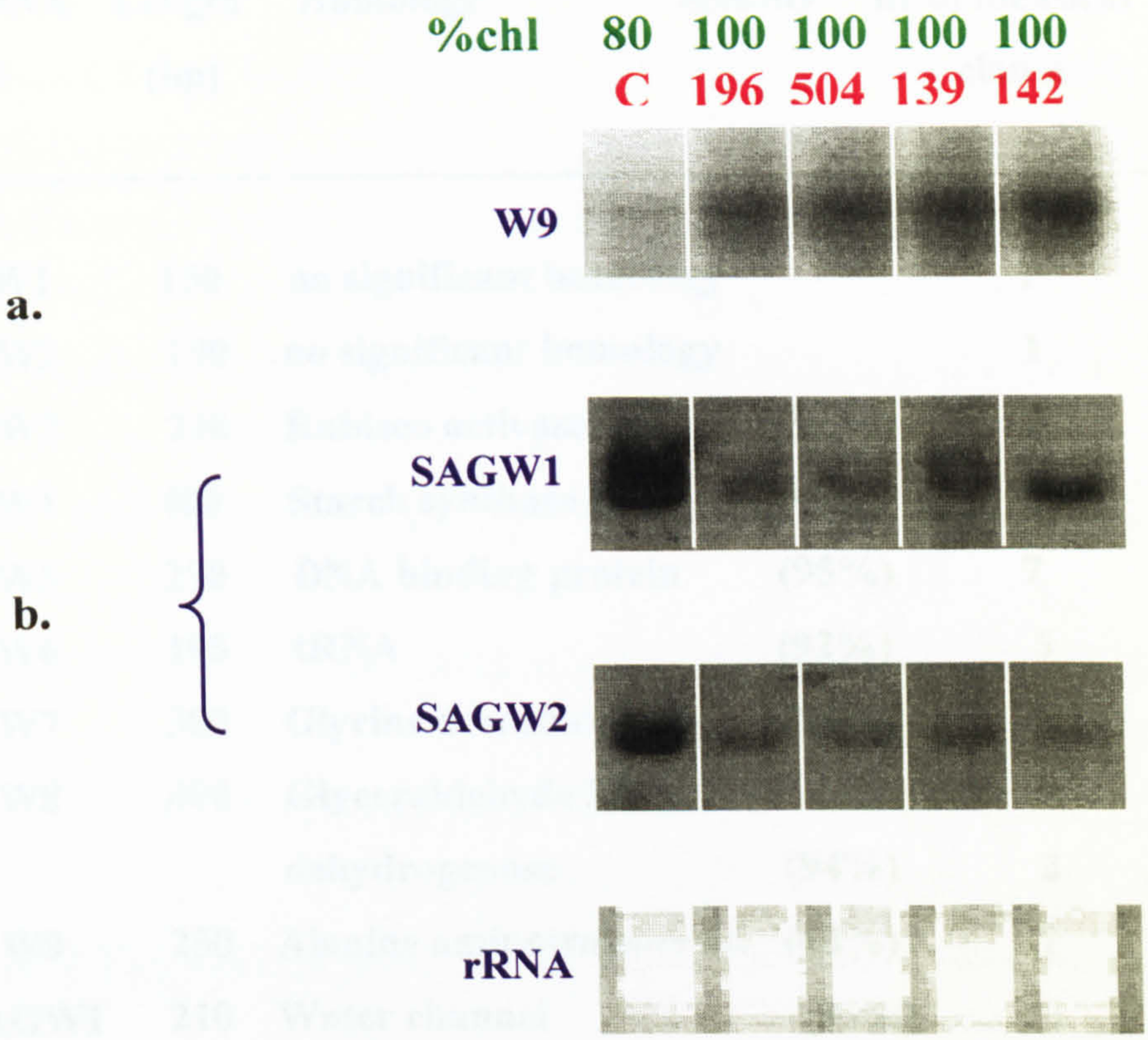


Fig. 4.4. Northern blot analysis of W9 (a), SAGW1 and SAGW2 (b) fragments cloned by the DDRT-PCR technologies.

On the base of the chlorophyll concentration the RNA was isolated from F1 stage, separated by electrophoresis and transferred to nylon membranes by northern blotting. 12 μg of RNA was loaded in each track. The rRNA was used as loading control.

C=control plant.

cDNA	Length (bp)	Homology	identity	nr of identical clones	Total clones	GenEMBL database
W1	150	no significant homology		1		
W2	190	no significant homology		1		
W3	240	Rubisco activase	(35 %)	2	3	HVRCABG
W4	400	Starch synthase I	(95%)	2	3	TA48226
W5	290	DNA binding protein	(95%)	7	8	TAHMGW
W6	195	tRNA	(92%)	5	6	TRANRN
W7	380	Glycine decarboxylase	(94%)	1	1	AF024589
W8	400	Glyceraldehyde 3P dehydrogenase	(94%)	2	3	X75326
W9	250	Alanine aminotransferase	(40%)	1	2	HVALAAT
SAGW1	210	Water channel	(35%)	1		X75883
SAGW2	210	no significant homology		1		
SAGW12	300	no significant homology		1		
SAGW14	310	no significant homology		1		

Table 4.1. Lengths of 32 fragments obtained by DDRT-PCR and homologies determined by screening sequence database.

DNA sequencing was carried out as described in Section 4.1.19. and the DNA sequences were analysed and compared with the GenEMBL databases using both the FASTA program or the BLAST network service (NCBI).

5' AGGCAGACCCTCTCATTCTCTTTTATTTTTAAATTTTACTAATCTCTTTA

TCCGTCTGGG.....

GTAAGACAGCCAAGGGACATACAGAAGCATATACCGAGATACAGATCTC

CAGATCACAGATTAAATGGGAGAGTTTCTGATGTGACACACCTTCCCAT

GGTGCACACATCATTTTATTCCGTCGAGCCGGGGCGGATCATTCTCCTT

TCCCCTTGGCATCCANTACCAAGCCTTCTCTGTACTCGTACTCCTGGCA

AAAAGTTTTNCTGGGGCCAATTTCAACTTTGCCACCTTGGCATTGACCC

ACCTTGGATCCAGCTGGCCATGCCTTTNTTTCGGGGAACGCGCTNCGGC

CAACAAAAAAAAAAAAAAA 3'

.....GTTTTTTTTTTTTTTT

Fig. 4.5. Partial sequence of one cDNA clone (W1) obtained by the DDRT-PCR technologies. The primers used for DDRT-PCR are underlined.

4.2.2.1 Starch synthase, glycine decarboxylase and glyceraldehyde 3-P dehydrogenase gene expression.

The coding sequence of **W4** (≅400 bp) shows significant homology to the sequence of the soluble starch synthase gene from *Triticum aestivum* **(Fig.4.6.)**.

Both starch and sucrose are synthesised from the triose phosphate that is generated by Calvin cycle (Beck and Ziegler 1989). Under high light conditions, the rate at which the chloroplasts supply photosynthate may exceed the ability of the cell to export the sucrose that is synthesised from that photosynthate. Under these conditions, starch is synthesised from the excess carbon. As the light intensity declines and night approaches, the supply of photosynthate decreases and, in turn, the rate of sucrose synthesis is reduced; as result, sucrose export exceeds the rate of sucrose synthesis **(Fig.4.7.)**. Therefore, much of the starch in the chloroplast is broken down at night to provide energy to the plant. Multiple isoenzymes of soluble starch synthase exist, but their specific functions are not known.

The coding sequence of **W7** (≅400 bp) shows significant homology to the sequence of the Glycine decarboxylase (P-protein) gene from *Hordeum* x *Triticum aestivum* **(Fig.4.8.)**.

Glycine decarboxylase is one of the key enzymes implicated in the photorespiratory carbon oxidation cycle (PCO) or C_2 cycle. The PCO cycle involves a cooperative interaction among three separate subcellular organelles (chloroplasts, peroxisomes and mitochondria), acting as a scavenger operation to recover the fixed CO_2 lost by oxygenase reaction of Rubisco. Indeed, during the C_2 cycle, two molecules of phosphoglycolate are converted to one CO_2 and one molecule of 3-phosphoglycerate (3-PGA), which can return to the C_3 cycle. Therefore, in C_3 plants the C_3 and C_2 cycles operate together in an integrated fashion and not as separate, independent pathways **(Fig.4.9.)**.

Glycine decarboxylase is a very abundant and complex enzyme that catalyses the oxidative decarboxylation of glycine to produce substrates such as CO_2 and NADH. The complex consists of four enzymes, including a pyridoxal phosphate (PLP)-containing protein (P-protein), a lipoamide containing protein (H-protein), a protein that interacts

with tetrahydrofolate (T-protein), and a FAD-containing lipoamide dehydrogenase (L-protein). The P-protein catalyses an oxidative decarboxylation of the glycine and transfers the reducing equivalents to the lipoamide on the H-protein (**Fig 4.10.**).

The coding sequence of the **W8** (≅400 bp) fragment shows significant homology to the sequence of glyceraldehyde 3-P dehydrogenase gene from maize (**Fig.4.11.**). Glyceraldehyde 3-P dehydrogenase catalyses a reaction in glycolysis in which the oxidation of the substrate (glyceraldehyde 3-phosphate) is linked directly to reduction of NAD^+ to NADH (**Fig. 4.12.**).

Glycolysis, the first stage of respiration, is a series of reactions carried out by a group of enzymes located in the cytosol. During this process, glucose, a six carbon sugar, is partly oxidised to produce two molecules of pyruvate (a three-carbon compound), ATP, and stored reducing power in the form of a reduced pyridine nucleotide, NADH. The subsequent oxidation of NADH by oxygen via the electron transport chain then releases sufficient free energy to drive the synthesis of ATP. Therefore, during respiration, free energy is released and incorporated into a form, ATP, that can be readily utilised for the maintenance and development of the plant.

The expression of genes enconding the soluble form of starch synthase, glycine decarboxylase (P-protein) and glyceraldehyde 3-P dehydrogenase, was determined by northern analysis in leaves from F1 stage of both mutant and control plants. At this stage, all the three genes examined, showed significantly higher levels of expression in leaves of mutant lines compared with those of control plants (**Fig.4.13.**). As senescence progresses, there is degeneration of the chloroplast, and the photosynthetic apparatus dismantled (Matile *et al.*, 1992). Because the senescence process had already started in control plants (chlorophyll concentration was 80% of the initial content), is not surprising that photosynthesis-related genes showed lower expression in control plants when compared with mutant lines.

The higher expression levels of photosynthesis-related genes in mutants plants may not be directly determined by the mutation(s), but may be a consequence (i.e. pleiotropic effect) of the extension of green leaf area which is sustained by functional photosynthesis.

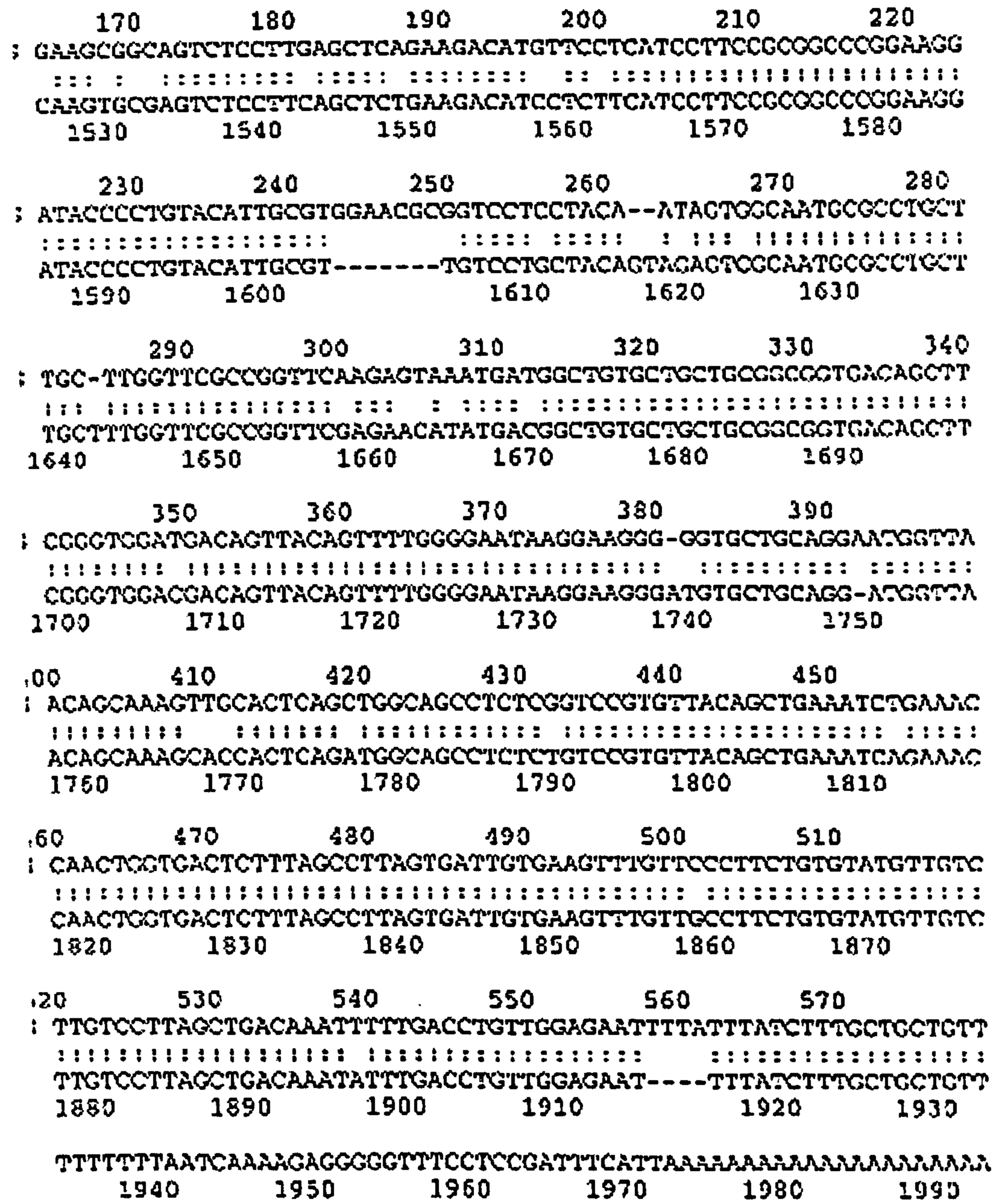


Fig. 4.6. Partial nucleotidic sequence of the W4 clone and alignment with the soluble starch synthase gene from *Triticum aestivum* (genBank/EMBL database TA48226).

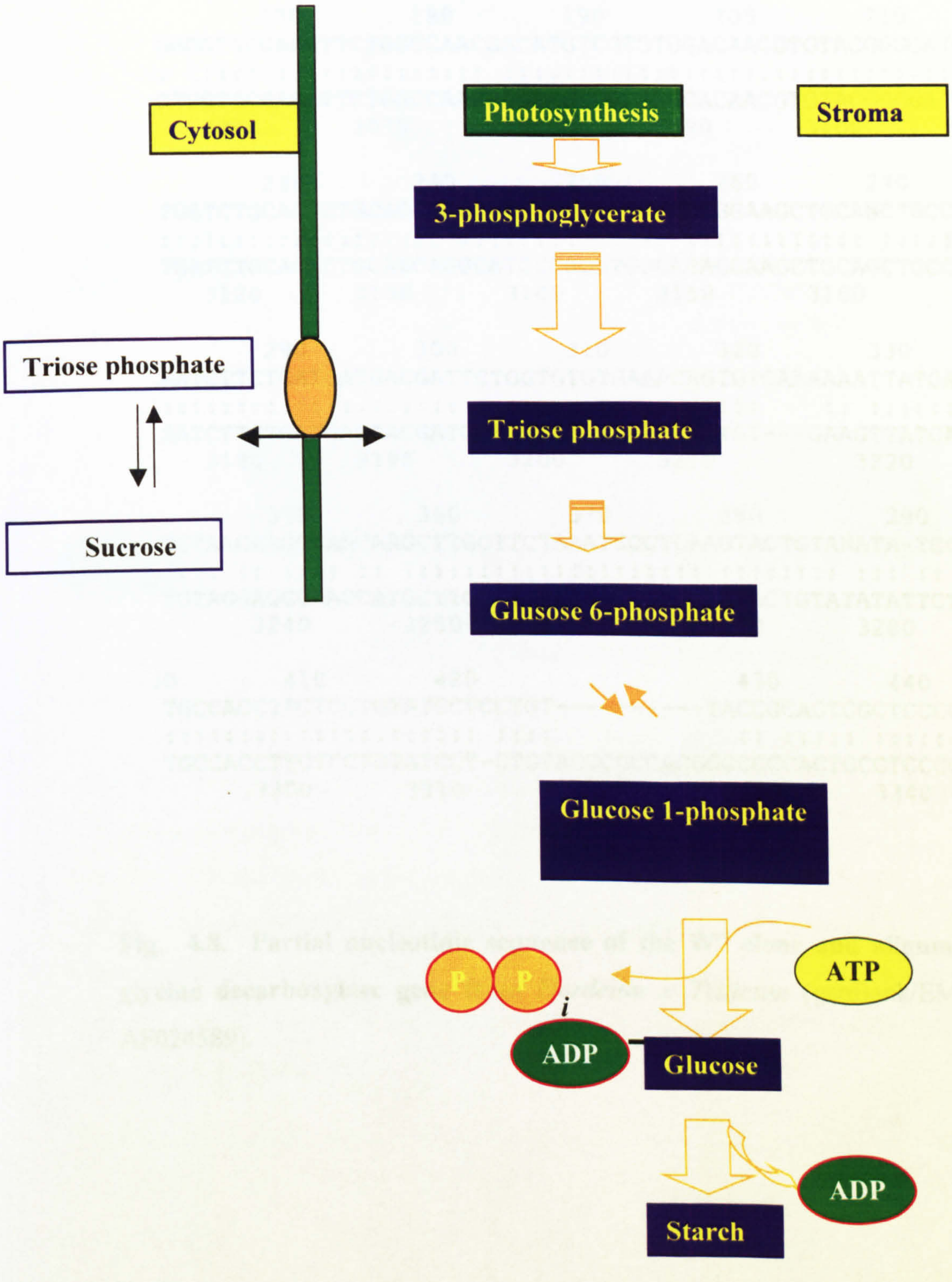


Fig 4.7. Synthesis of starch and sucrose from the triose phosphate generated by Calvin cycle.

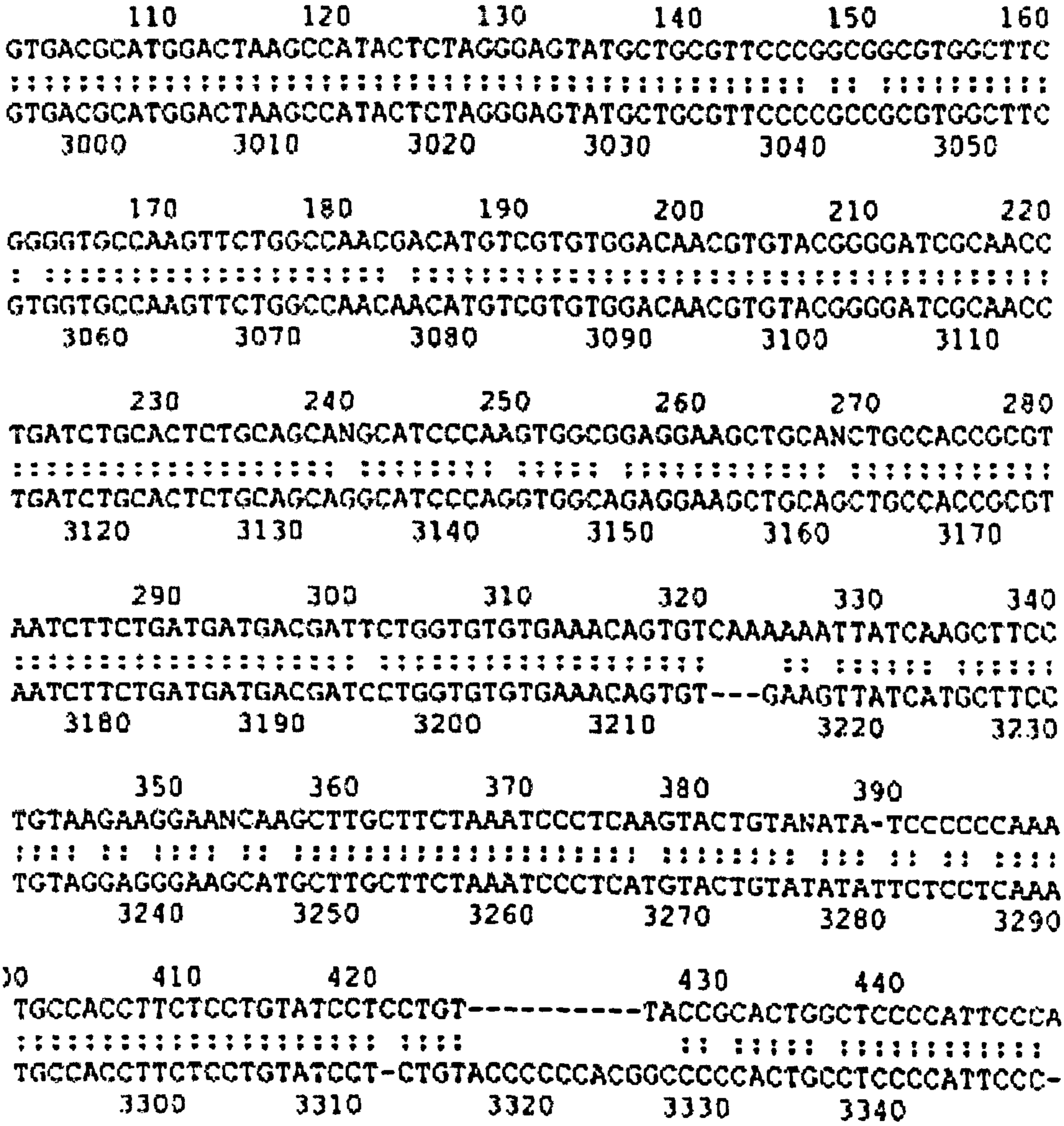


Fig. 4.8. Partial nucleotidic sequence of the W7 clone and alignment with the glycine decarboxylase gene from *Hordeum x Triticum* (genBank/EMBL database AF024589).

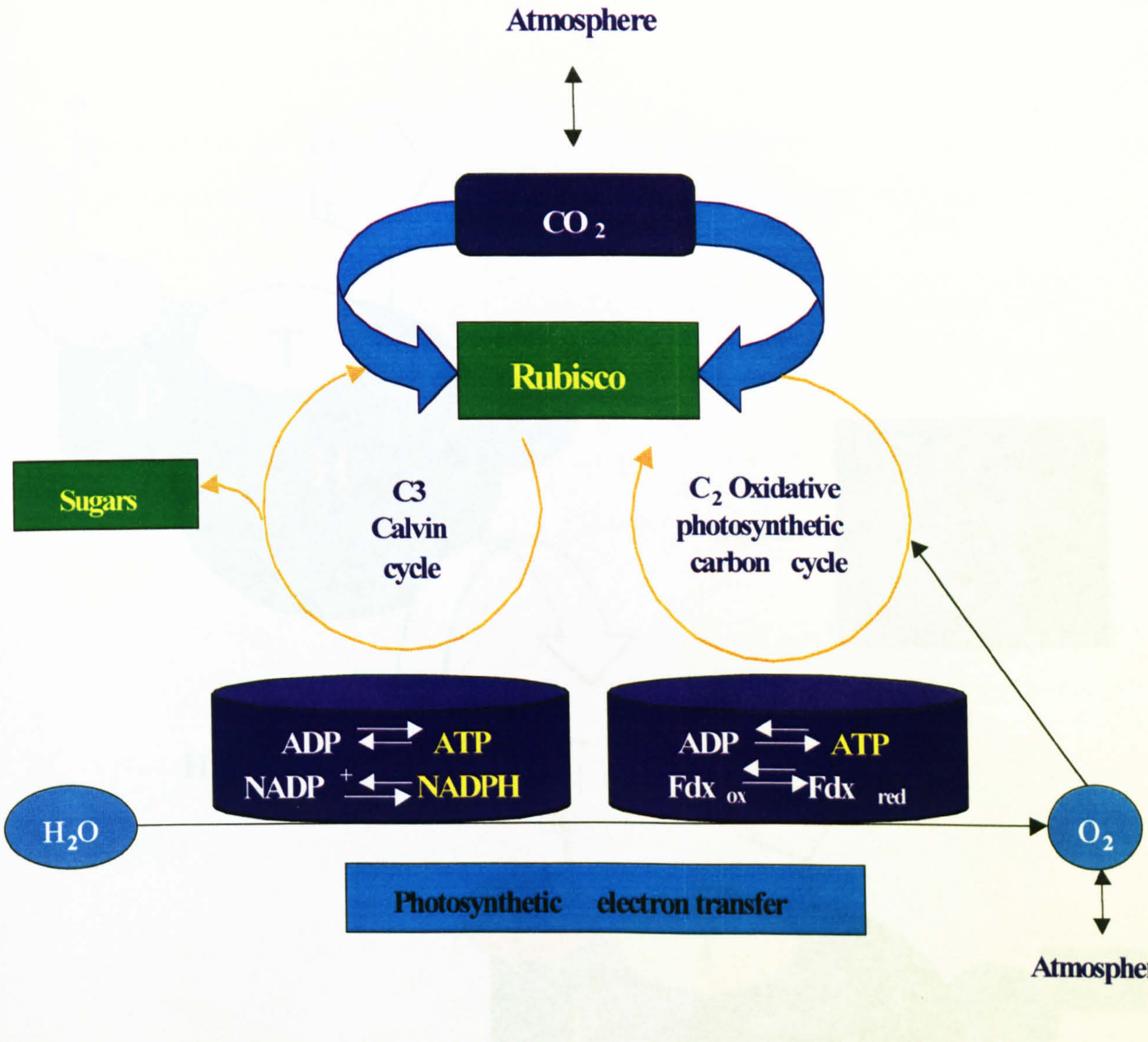


Fig. 4.9. Diagram of the relationship between the reductive photosynthetic (C_3) and the oxidative photosynthetic (C_2) carbon cycles.

Rubisco initiates both the Calvin cycle and the photorespiration (the C_2 Cycle). In both cases, photosynthetic electron transport provides energy-rich substrates.

One of the substrates of the C_3 cycle, CO_2, is a product of the C_2 cycle; in turn, the substrate of the C_2 cycle, O_2, is a product of C_3 photosynthesis (adapted from Siedow, J.N., and Day, D.A., 2000).

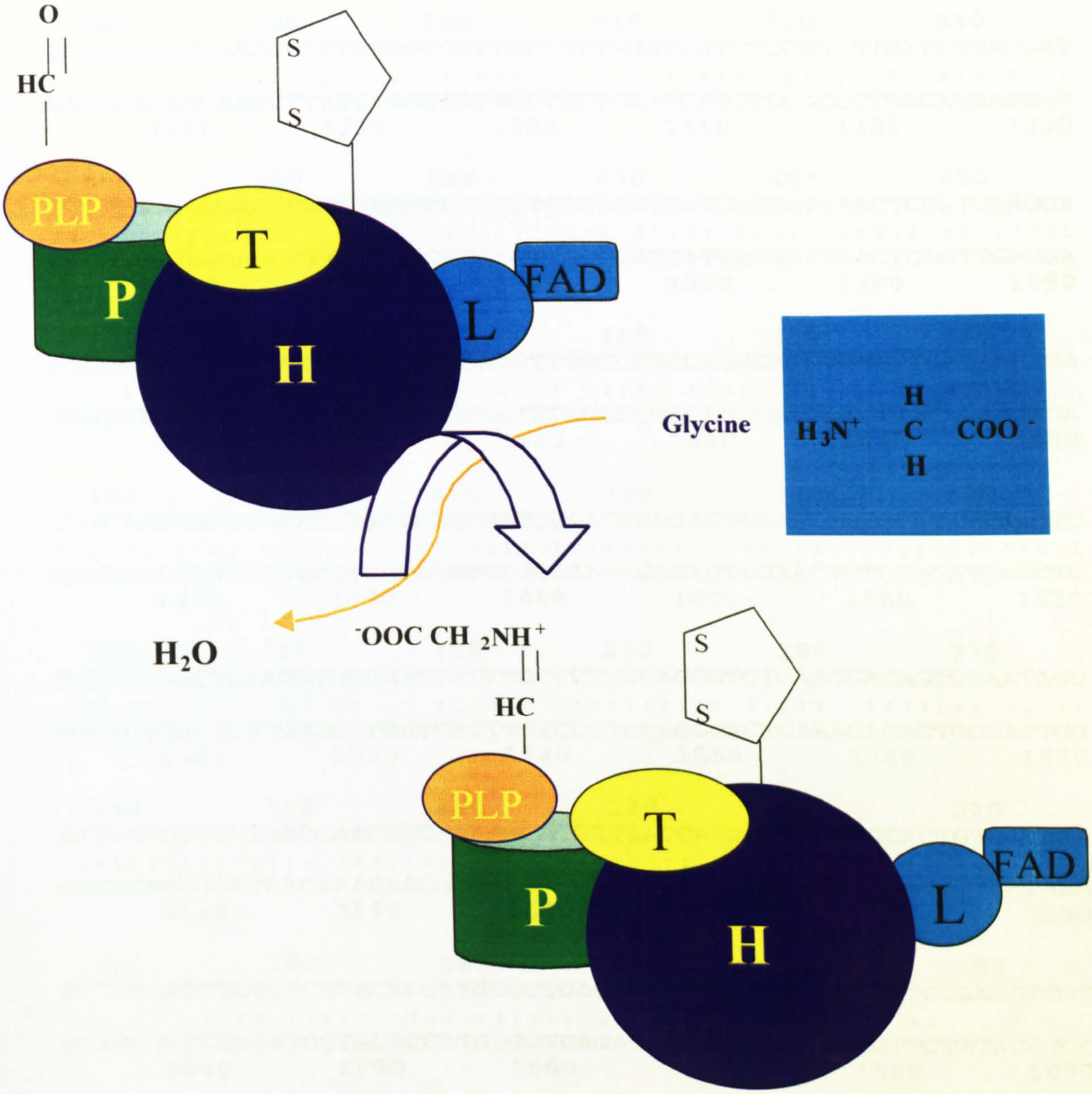


Fig. 4.10. Reaction mechanism of the glycine decarboxylase complex.

The reaction sequences is initiated by formation of a Schiff's base between glycine and the PLP on the P-protein. The P protein catalyses an oxidative decarboxylation of the glycine and transfers the reducing equivalents and the remaining.

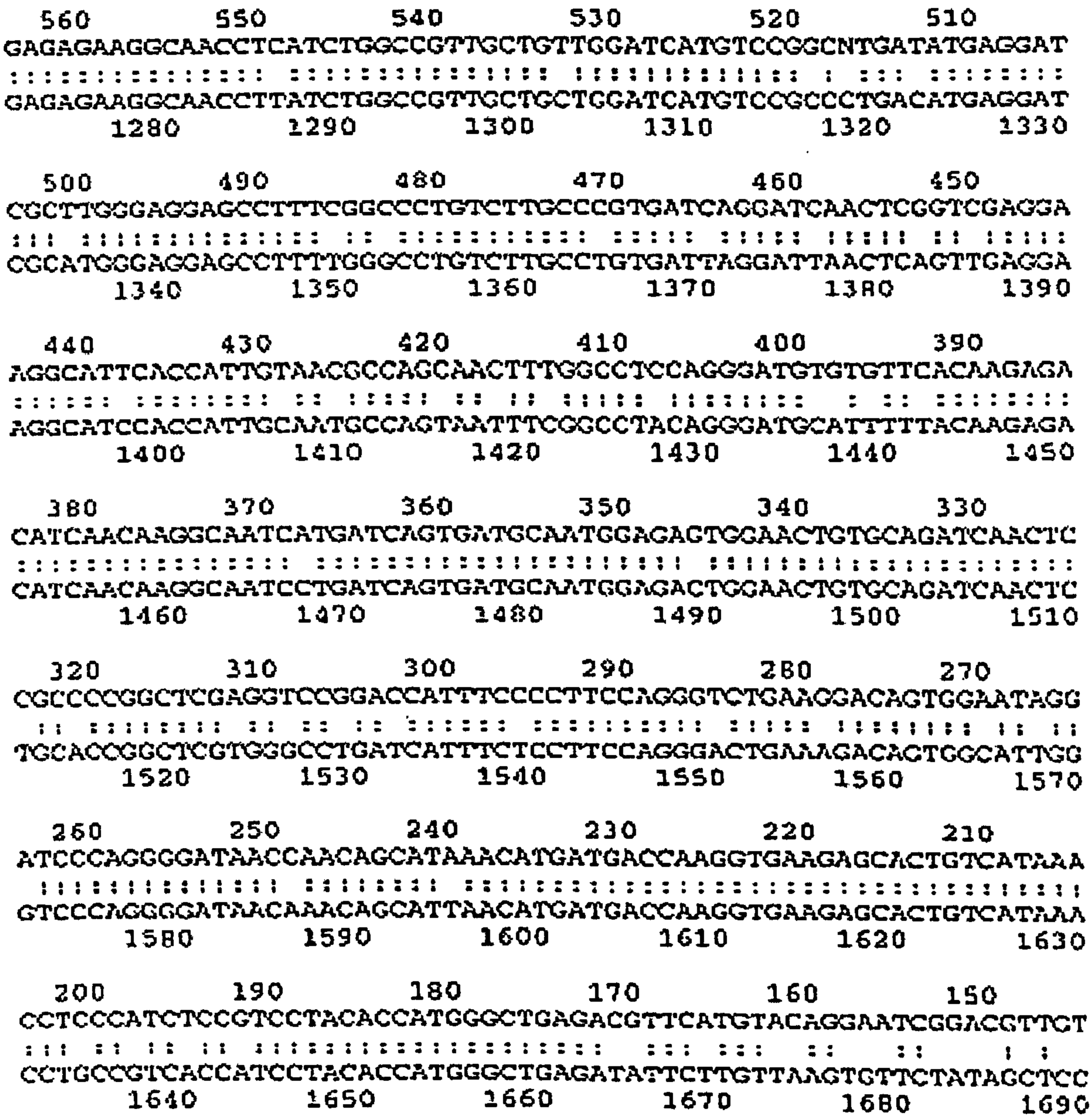


Fig. 4.11. Partial nucleotidic sequence of the W8 clone and alignment with the glyceraldehyde 3P dehydrogenase gene from maize (genBank/EMBL database X75326).

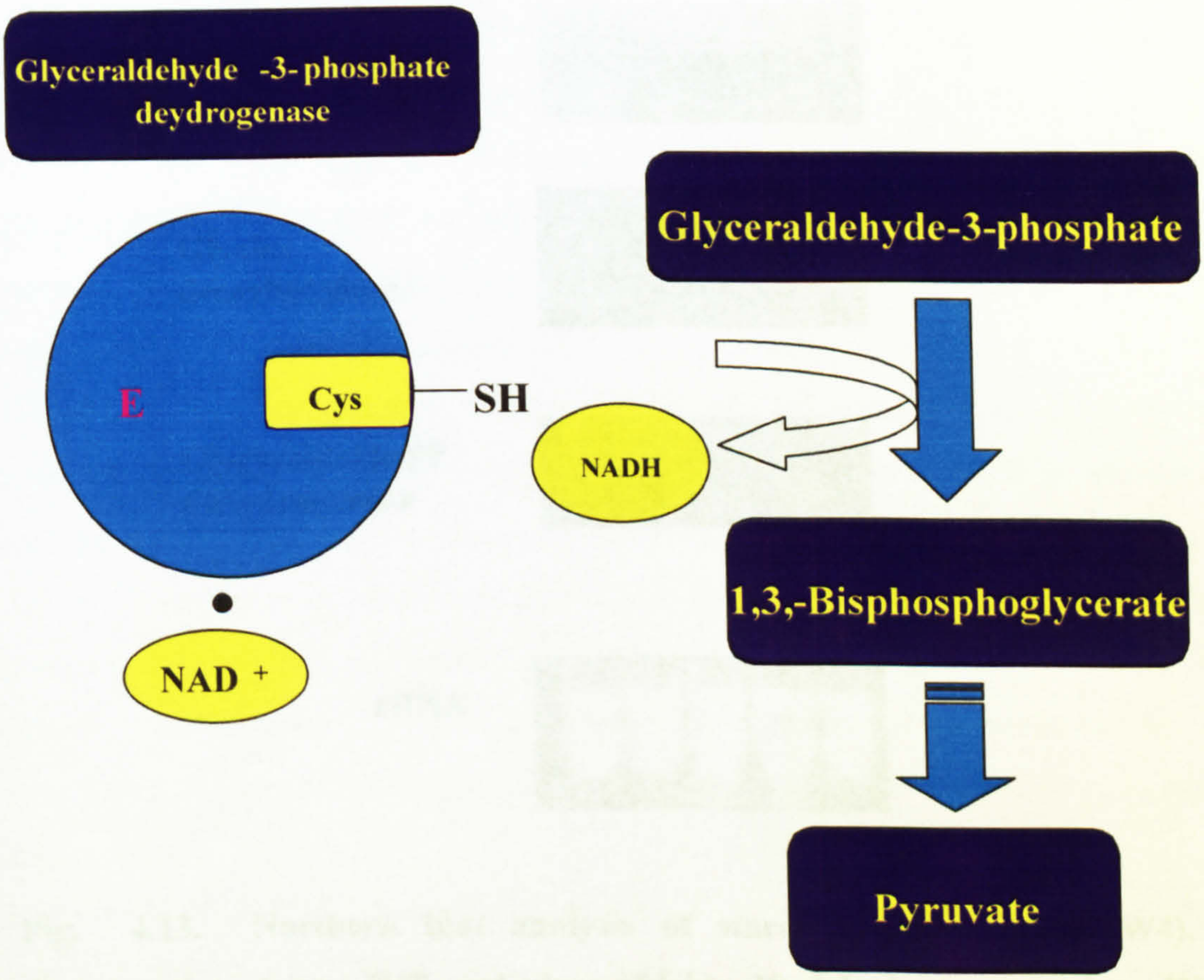


Fig.4.12. Oxidative reaction of Glyceraldehyde 3-P dehydrogenase.

Glyceraldehyde 3-P dehydrogenase catalyses a reaction in glycolysis in which the oxidation of the substrate is linked directly to reduction of NAD^+ to NADH.

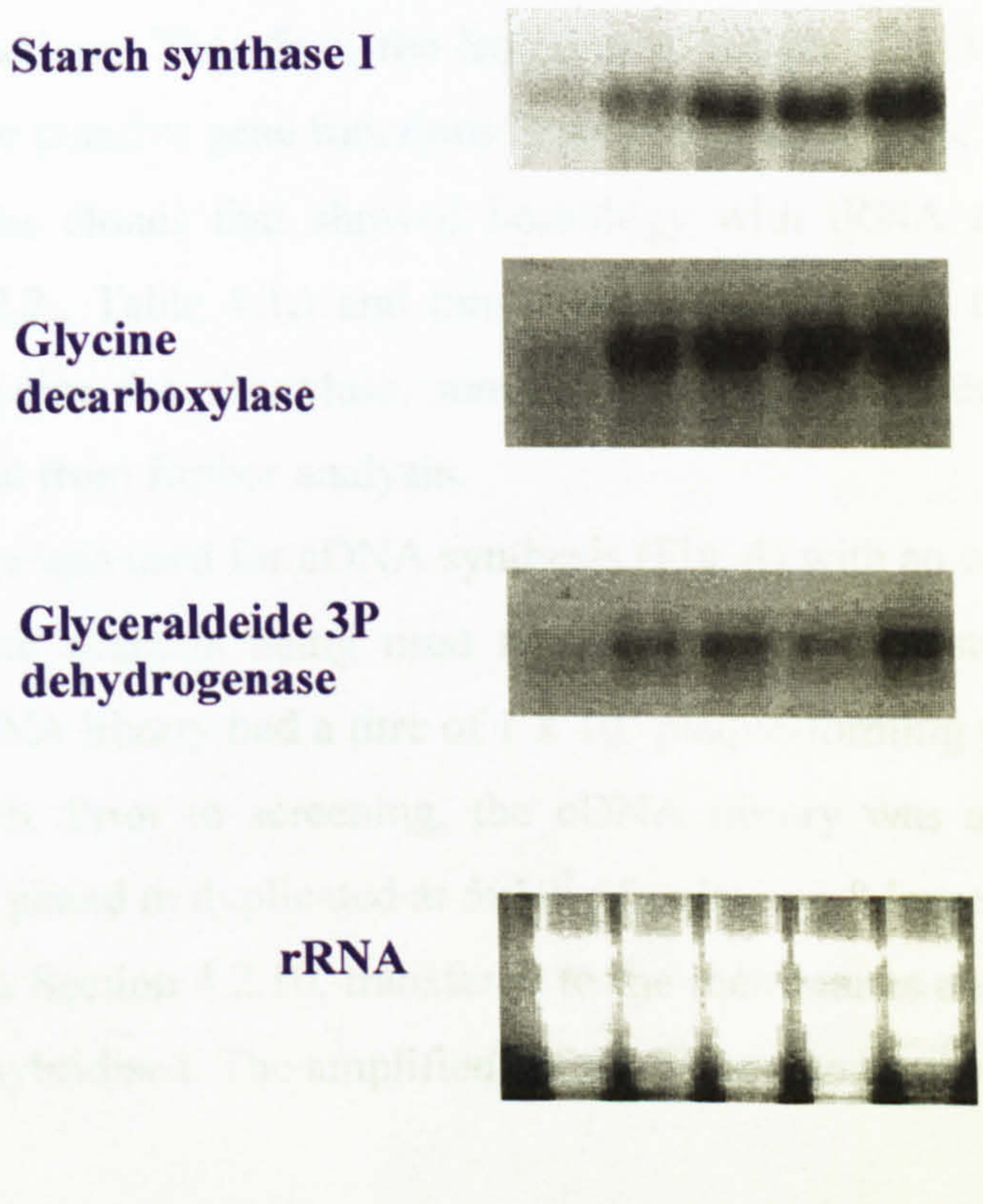


Fig. 4.13. Northern blot analysis of starch synthase (clone W4), glycine decarboxylase (clone W7) and glyceraldeide 3P dehydrogenase (clone W8) genes cloned by the DDRT-PCR technologies.

On the base of the chlorophyll concentration the RNA was isolated from F1 stage, separated by electrophoresis and transferred to nylon membranes by northern blotting. 12 μg of total RNA was loaded in each track. The rRNA was used as a loading control. **C=control plant.**

4.2.3. Construction of a cDNA library, screening and sequence analysis of cDNA clones.

Because cDNAs cloned by DDRT-PCR are generally short (150-350bp) and represent the 3' region of the gene, the homology with known genes may be too low to assign putative functions. Therefore, the isolation of longer cDNAs may be necessary before searching for putative gene functions in sequence data bases.

However, the clones that showed homology with tRNA and DNA binding proteins (Section 4.2.2., Table 4.1.) and those that showed strong homology with other genes (such as glycine decarboxylase, starch synthase I and glyceraldeide 3P deydrogenase) were omitted from further analysis.

The F1 stage was used for cDNA synthesis (Fig. 4) with an equimolar aliquot of mRNAs from all four mutants being used as template for first strand cDNA synthesis. The primary cDNA library had a titre of 1×10^6 plaque-forming units, with an average insert size of 1 kb. Prior to screening, the cDNA library was amplified and the amplified library was plated in duplicated at 5×10^5 pfu/plate on 8 large 24 x 24 NZY agar plates as described in Section 4.2.10, transfered to the membranes and the duplicated membranes were then hybridised. The amplified cDNA library had a titre of 2×10^{11} plaque-forming units.

The seven clones that showed either no homology or only weak homology with known genes were used as probes for library screening. The probes were labelled separately and a mix of differentially expressed fragments (four and three respectively) was used for each duplicate membrane.

Three rounds of screening were performed and the cDNA clones obtained by plaque screening were sequenced.

The results of the homology search are presented in **Table 4.2.** The sequences were also compared with those obtained by DDRT-PCR in order to confirm that they corresponded to the original clones.

Fig. 4.14. gives a brief outline of the procedure.

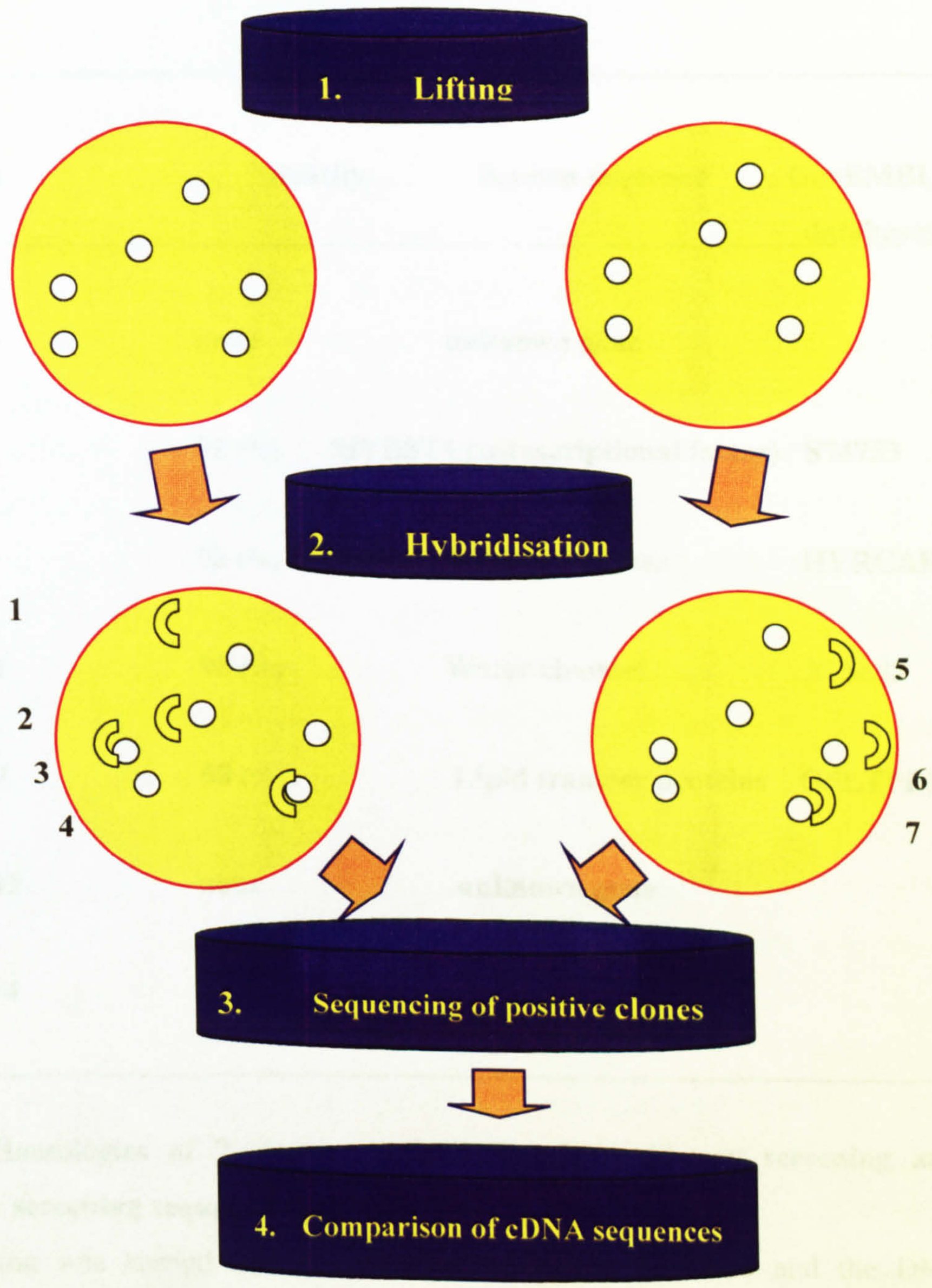


Fig.4.14. A brief outline of the procedure for screening the cDNA library and recovery of positive clones

Each step is described in Section 2.2.

cDNA clone	Identity	Known sequence	GenEMBL databases
1. W1	none	unknown gene	
2. W2	92 (%)	MYBST1 (transcriptional factor)	S74753
3. W3	93 (%)	Rubisco activase	HVRCABG
4. SAWG1	98 (%)	Water channel	X75883
5. SAWG2	58 (%)	Lipid transfer proteins	OSLTPB1
6. SAWG12	none	unknown gene	
7. SAWG14	none	unknown gene	

Table 4.2. Homologies of 7 clones obtained by cDNA library screening and determined by screening sequence database.

DNA sequencing was carried out as described in Section 2.1.12.20 and the DNA sequences were analysed and compared with the GenEMBL databases using both the FASTA program or the BLAST network service (NCBI).

The coding sequence of the **W3** (≅700 bp) clone shows significant homology to the nucleotide sequence of the rubisco activase gene from barley (confirming as previously reported in table 5) **(Fig.4.15.)**.

Rubisco activase normally accumulates in greening or photosynthetic tissues expressing Rubisco. The primary role of Rubisco activase is to reactivate rubisco that has been deactivated by sugar biphosphates **(Fig.4.16.)**. Rubisco activase accelerates the release of bound sugar biphosphates that inhibit the decarbamylated form of Rubisco and also removes carboxyarabinitol-1-phosphate, a rubisco inhibitor. The inhibitor binds to Rubisco at night and is removed in the morning, when photon flux density increases. Hence, it has a regulatory role in photosynthetic C reduction via the action of Rubisco.

The expression of the Rubisco activase gene was determined by northern analysis in leaves from F1 stage of both mutant and control plants. The gene showed significantly higher levels of expression in leaves of the mutant lines compared with those of the control plants **(Fig.4.17.)**. Degradation of rubisco has been reported to be an early event in the senescence-specific dismantling of chloroplasts (Evans, 1988). Therefore, the higher expression of Rubisco activase gene in leaves of the mutant lines may suggest (as already shown for starch synthase, glycine decarboxylase and glyceraldehyde 3-P dehydrogenase) that the extension of the green leaf area in mutants plants is sustained, even at the molecular level, by functional photosynthesis.

The coding sequence of the **W2** (≅700 bp) clone shows significant homology to the nucleotide sequence of **mybSt1** a **Myb**-related transcription factor from *Solanum tuberosum* **(4.18.)**.

An important difference between transcription in prokaryotes and eukaryotes is that the RNA polymerases of eukaryotes require additional proteins called general transcription factors to position them at the correct start site. General transcription factors make up a large, multisubunit transcription initiation complex. MYBs are transcription factors that enhance the rate of transcription by faciliting the assembly of the transcription initiation complex. Usually, MYBs regulate growth and development. Recently, a MYB-related transcriptional factor was characterised in *Arabidopsis* (Wang *et al.* 1997). The mRNA

level increased rapidly when *Arabidopsis* was transferred from the dark to the light and the protein appears to bind to the promoter of the light-harvesting chlorophyll a/b-binding protein (LHCP) and regulate its transcription (Wang *et al.* 1997).
However, in the present case, further experiments needed to assign any function to the gene.

Unlike the expression of the photosynthesis_related genes, the **SAWG1** and **SAWG2** genes showed enhanced expression in leaves of control plants but were less easily detected at F1 stage in the mutant lines (see Fig.4.4.a and 4.4.b).
Because the senescence process had already started in the samples of control plants taken at the F1 stage [i.e. chlorophyll content was 80% of the initial content (see Fig.4)], the SAWG1 and SAWG2 genes could represent senescence_related genes whose expression was increased during leaf senescence. Because these genes were less readily detectable in mutants plants, these results may suggest a delay in the onset of the senescence process.

The coding sequence of the **SAWG1** (≅750 bp) clone shows significant homology to the nucleotide sequence of the plasma membrane-intrinsic protein (PIP) gene from *A. thaliana* **(Fig.4.19.)**.
Regulation of water transport between animal or plant cells involves specialised water-conducting proteins, called aquaporins, which possess water-channel activity. Aquaporins are members of the major intrinsic protein (MIP) superfamily including the plasma membrane-intrinsic protein (the PIP subfamily) and the salt-stress inducible tonoplast intrinsic protein (the TIP subfamily). These proteins are highly expressed not only in developing but also in mature leaves which export photosynthates (Frangne *et al.*, 2001). Furthermore, they may be involved in the osmoregulation in plants under high osmotic stress such as under high salt condition (Pih *et al.*, 1999).
Recently, a MIP-homologus protein (TRAMP–tomato ripening associated protein) has been shown to be induced by ethylene in ripening fruit and senescing leaves (Fray *et al.*, 1994). Although it is difficult to propose a role for water channel in senescing leaves, either TRAMP or SAGW1 may acts as transporter of small molecules, and senescing leaves are net exporters of many compounds.

The coding sequence of the **SAWG2** (≅750 bp) clone shows homology to the nucleotide sequence of the lipid transfer protein gene from rice *Oryza sativa* **(Fig.4.20.)**.

Plant cells contain cytosolic proteins, called lipid transfer proteins (LTP), which are able to facilitate intermembrane transfer of phospholipids *in vitro*. Comparison of their amino acid sequences had revealed striking homologies and conserved domains which are probably involved in their function. These proteins could play a major role in membrane biogenesis. Indeed, it has been proposed that lipids synthesised in the ER could be removed from the membrane by a lipid-transfer protein and deposited in the outer envelope of the chloroplast. However, there is no direct evidence for their real function. Recently, plant lipids transfer proteins, have been identified as important food allergens (Pastorello *et al.*, 2001).

During senescence the membranes of plant cells constitute a valuable store of lipid molecules which can be mobilised and used by the senescing leaf. The level of total lipids decreases in senescing leaves and it appears that the membranes of the cell, including the thylakoid membranes are metabolised to provide energy for the senescence process (Wanner *et al.*, 1991). The levels of key enzymes of the glyoxylate cycle, malate synthase and isocitrate lyase have been shown to increase in senescing leaves of barley (Gut and Matile, 1998). The glyoxylate cycle is involved in the conversion of acetyl CoA, produced after lipid breakdown, to four carbon acids for subsequent conversion to carbohydrates, particularly sucrose, by gluconeogenesis (**Fig. 4.21.**).

Therefore, the high expression of a gene encoding a putative LTP during leaf senescence could indicate a role for this protein in transfering membranes lipids to the site of degradation.

Scanning of the GenEMBL database with the **W1**(≅780 bp), **SAGW12**(≅820 bp) and **SAGW14** (≅1 kb) clones provided no significant similarities with published sequences. Therefore, it was impossible to suggest any function for these genes.

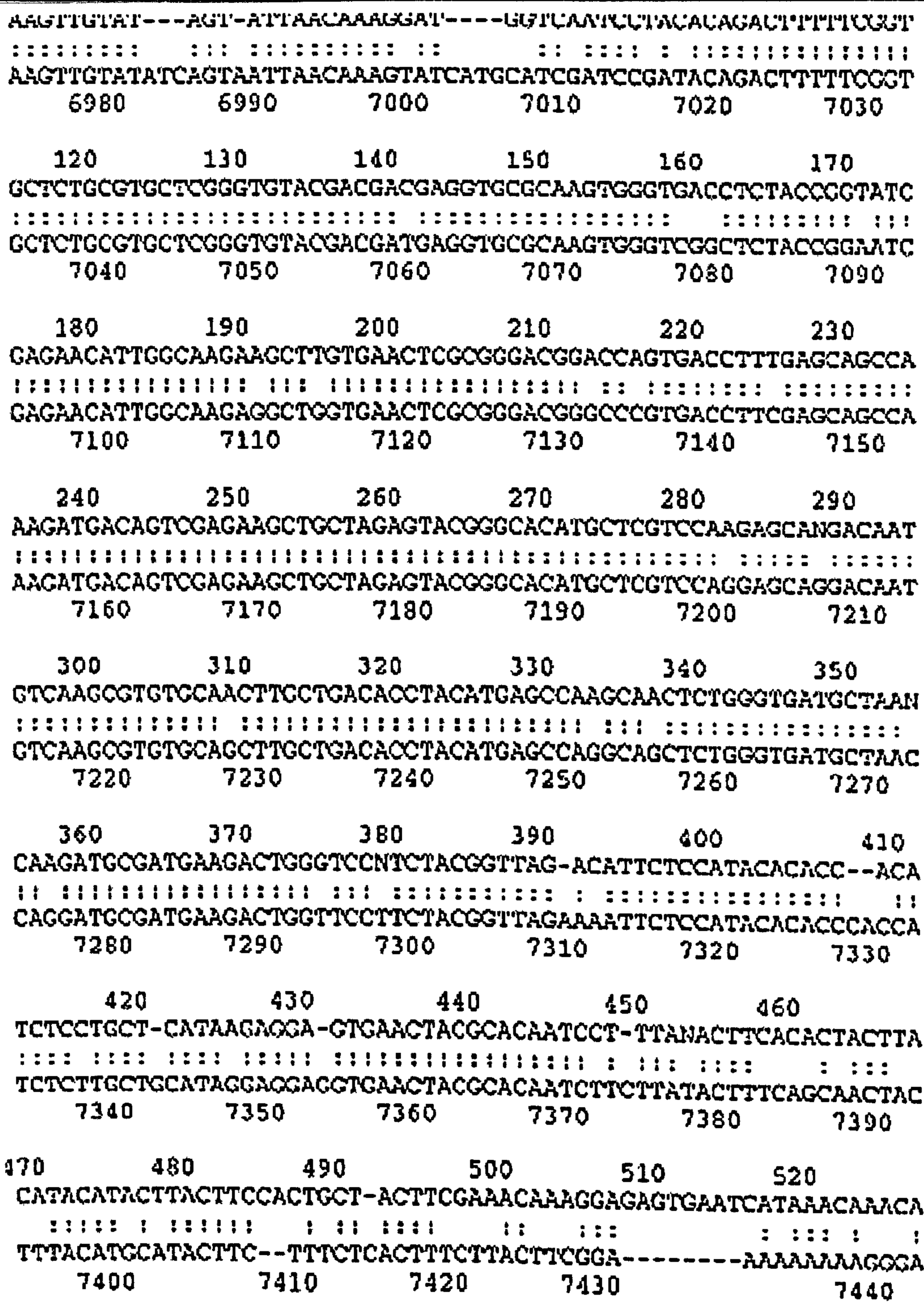


Fig. 4.15. Partial nucleotidic sequence of the W3 clone and alignment with the rubisco activase gene from *Hordeum vulgarum* (genBank/EMBL database HVRCABG).

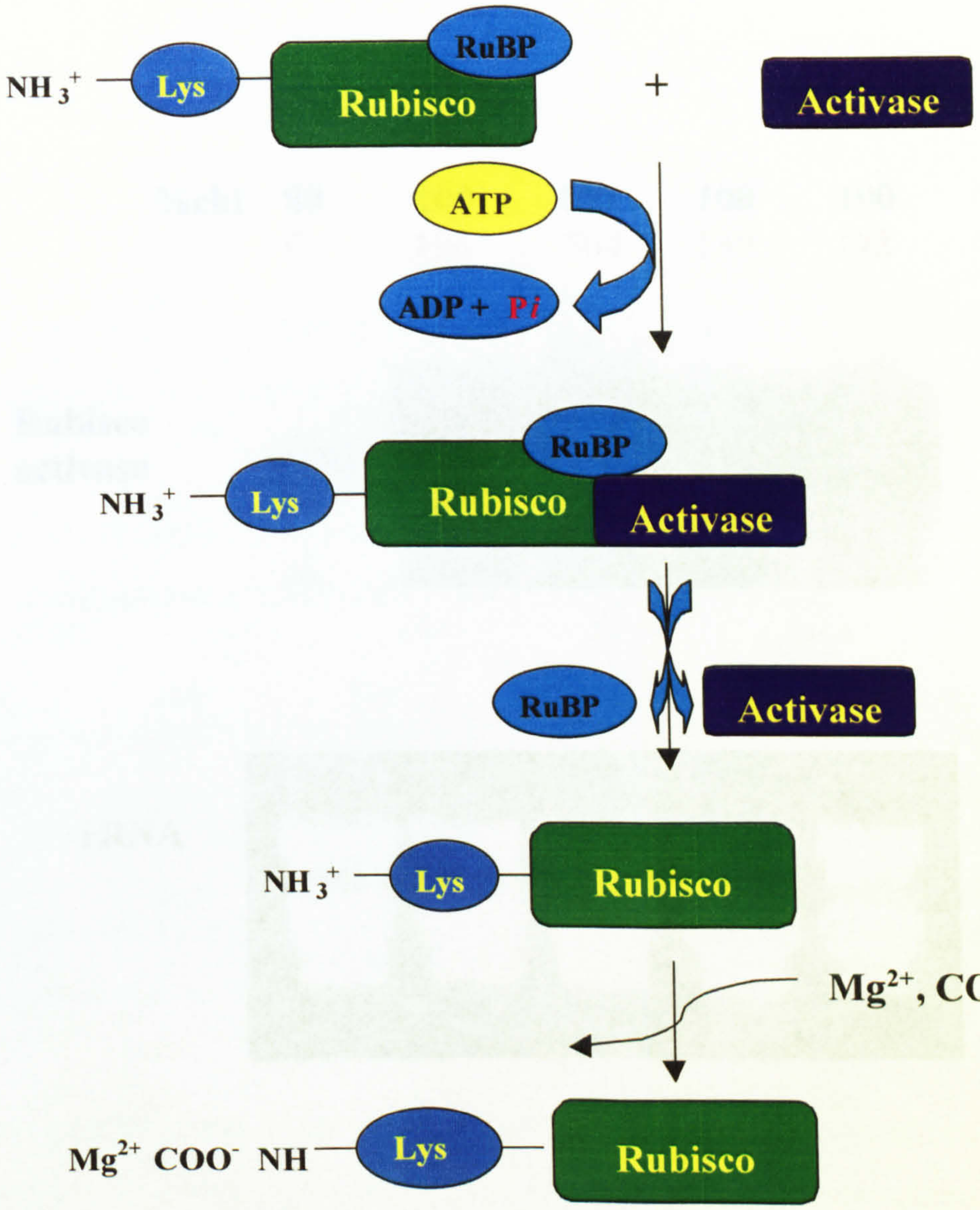


Fig.4.16. Regulation of Rubisco by rubisco activase.

Rubisco activase removes bound RuBP from inactive, decarbamylated rubisco in an ATP-dependent reaction. The free Rubisco can then be activated by carbamylation, binding CO_2 and Mg^{2+}.

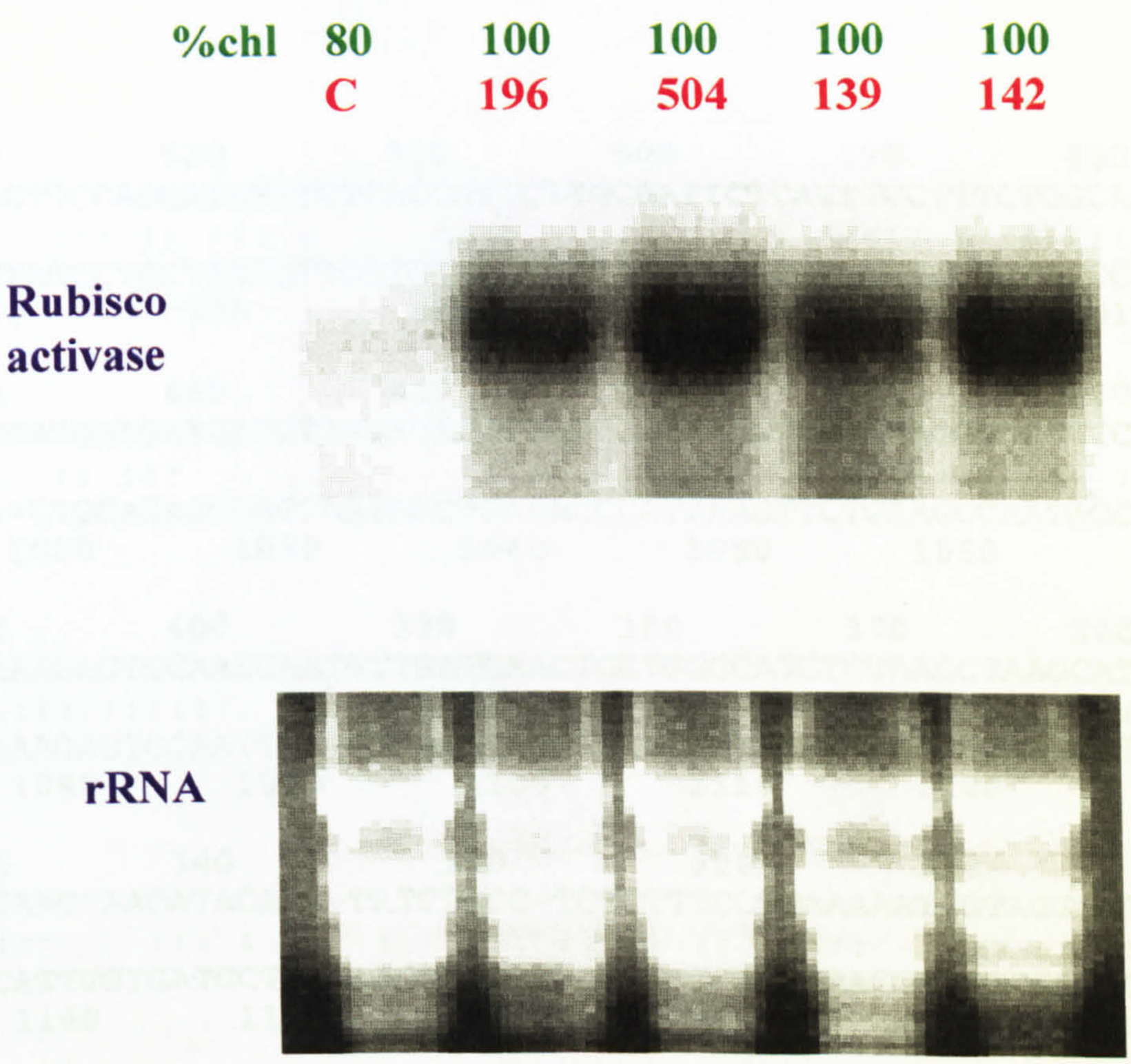


Fig. 4.17. Northern blot analysis of rubisco activase (clone W3), cloned by the DDRT-PCR technologies.

On the basis of the chlorophyll concentration the RNA was isolated from F1 stage, separated by electrophoresis and transferred to nylon membranes by northern blotting. 12 μg of total RNA was loaded in each track. The rRNA was used as a loading control. **C=control plant.**

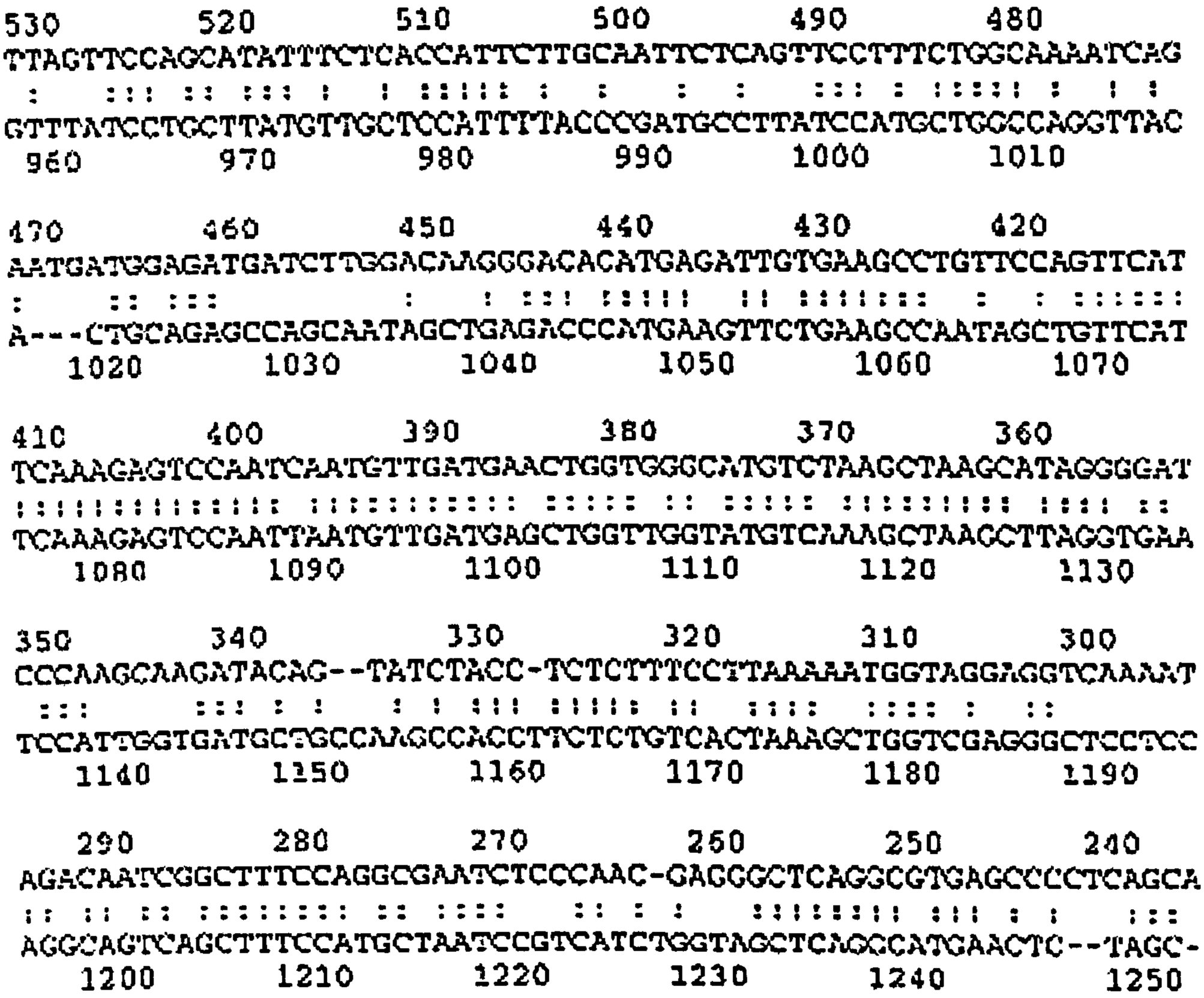


Fig. 4.18. Partial nucleotidic sequence of the W2 clone and alignment with MYBST1 a myb-related transcriptional factor from *S. tuberosum* (genBank/EMBL database S74753).

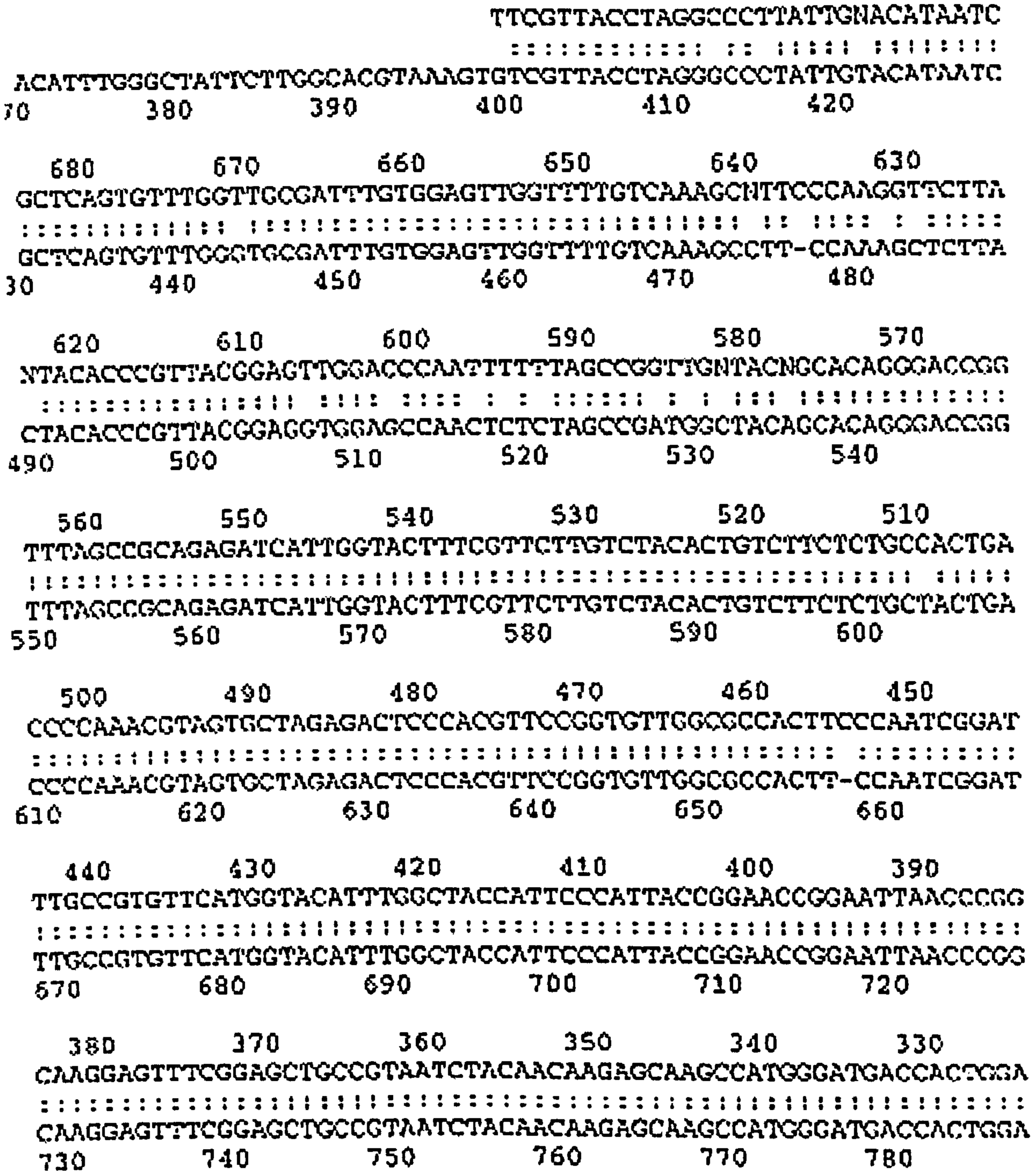


Fig. 4.19. Partial nucleotidic sequence of the SAGW1 clone and alignment with a plasma membrane intrinsic protein 2A from *A. thaliana* (genBank/EMBL database X75883).

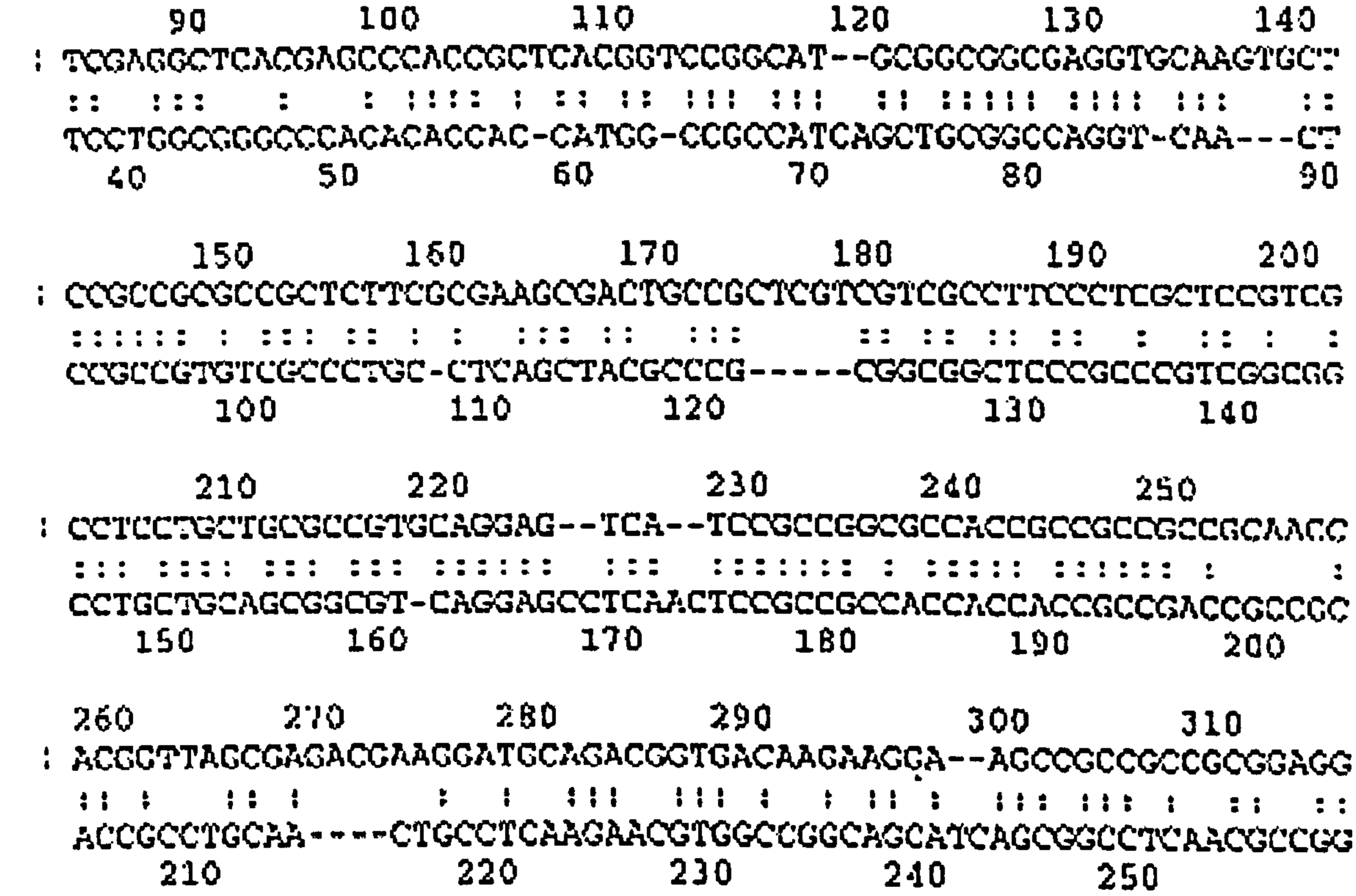


Fig. 4.20. Partial nucleotidic sequence of the SAGW2 clone and alignment with a lipid transfer protein from rice (*O.sativa*) (genBank/EMBL database OSLTPB1).

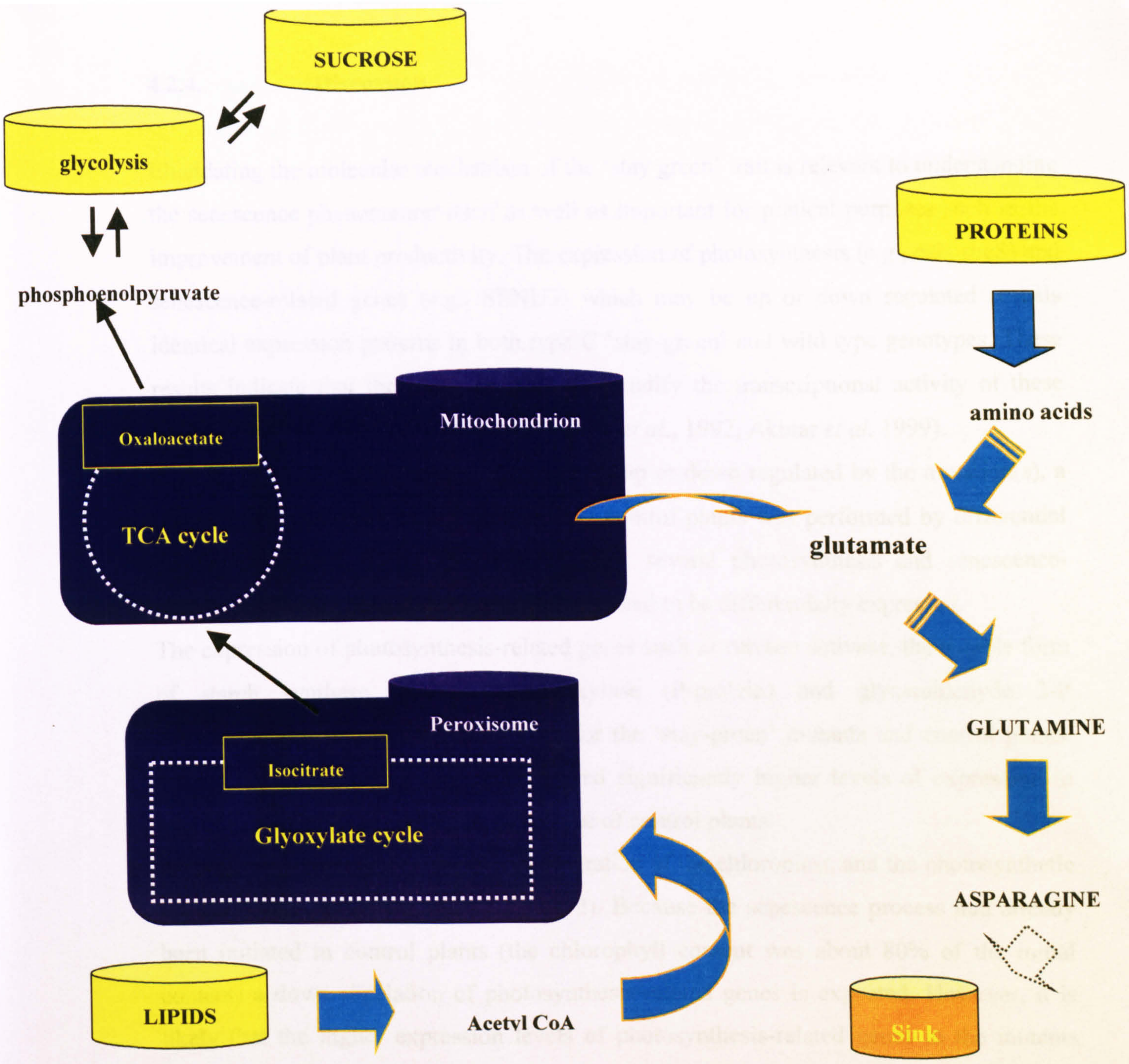


Fig.4.21. Possible metabolic scheme controlling senescence-related mobilisation of macromolecule components.

The succinate produced from the conversion of acetylCoA in the glyoxylate cycle can feed into the TCA cycle where it can be converted to oxaloacetate for gluconeogenesis. Sucrose, synthesised via gluconeogenesis, is either used for respiration or is exported. Glutamine and asparagine are transported from the senescing cell. The arrows indicate the direction which the processes are likely to take during senescence (adapted from Buchanan-Wollaston, 1997)

4.2.4. Discussion

Elucidating the molecular mechanism of the 'stay green' trait is relevant to understanding the senescence phenomenon itself as well as important for pratical purposes such as the improvement of plant productivity. The expression of photosynthesis (e.g., cab, rbcS) and senescence-related genes (e.g., SENU3) which may be up or down regulated reveals identical expression patterns in both type C 'stay-green' and wild type genotypes. These results indicate that the mutation does not modify the transcriptional activity of these genes during the senescence process (Thomas *et al.*, 1992, Akhtar *et al.* 1999).

In order to isolate genes whose expression is up or down regulated by the mutation(s), a comparative analysis between mutants and control plants was performed by differential display of mRNA. Using this methodology, several photosynthesis and senescence-related genes were cloned and were demonstrated to be differentially expressed.

The expression of photosynthesis-related genes such as rubisco activase, the soluble form of starch synthase, glycine decarboxylase (P-protein) and glyceraldehyde 3-P dehydrogenase, reveals different trends for the 'stay-green' mutants and control plants. Indeed, all of the genes examined showed significantly higher levels of expression in leaves of mutant lines compared with those of control plants.

As senescence progresses, there is degeneration of the chloroplast, and the photosynthetic apparatus dismantled (Matile *et al.*, 1992). Because the senescence process had already been initiated in control plants (the chlorophyll content was about 80% of the initial content) a down regulation of photosynthesis-related genes is expected. However, it is likely that the higher expression levels of photosynthesis-related genes in the mutants plants are not a direct effect of the mutation(s) but a consequence (i.e. pleiotropic effect) of the extension of the green leaf area which is sustained by functional photosynthesis.

Unlike the expression of the photosynthesis-related genes, the SAWG1 and SAWG2 genes show enhanced expression in leaves of control plants but were less easy to detect in the mutant lines.

Therefore, the SAWG1 and SAWG2 genes could represented senescence-related genes whose expression has been increased during leaf senescence. Because these genes are less readly detected in the mutant plants, these results may suggest a delay in the rate of progress of the senescence process.

Other genes such as W1, SAWG12, SAWG14 (unknown functions) and W2 (*myb* trancriptional factor) require further analysis in order to determining their functions.

Taken together, these results give a clear picture of the 'stay green' phenotype in durum wheat. The extended expression of photosynthesis-related genes and the lower expression of senescence-related genes confirm, at molecular level, that the novel mutants have the characteristics of functional 'stay green' according to Thomas and Smart (1993).

Chapter 5

Final Discussion, Conclusions and Future Work

5. Final Discussion, Conclusions and Future work

The different organs of plants have diverse biological roles and requirements. One of the crucial functions of source leaves is the synthesis of energy-rich carbon molecules which are transported to heterotrophic sink organs, such as developing fruits, seeds, roots **(Fig.5.)** The greater the ability of a sink to store or metabolise imported sugars (the process of allocation), the greater is its ability to compete for assimilates being exported by the sources. However, events in the sources and sinks must be synchronised. Therefore, the partitioning of photoassimilates between source leaves and different heterotrophic plant organs (sink organs) is a highly regulated process with several control points.

In cereals such as wheat the yield and percentage of protein are key issue for both the production and marketing of grains. Yield is a product of the activities of processes contributing to the deposition of starch in the grain while protein percentage reflects the processes of nitrogen metabolism. Starch and protein accumulate in the endosperm from precursors (sucrose for starch synthesis and amino acids for protein synthesis) supplied by the rest of the plant. Limits to the rate of starch deposition result from a balance between the capacity of the plant to produce substrate (source limited) and the capacity of the grain to utilise it (sink limited). However, an extension of the grain filling period by extending the period of photosynthetic activity during senescence may alter the rate and duration of both starch and protein deposition.

Furthermore, the protein deposited in the grain is derived partly from the protein in the leaf (particularly Rubisco) that is responsible for fixing CO_2 to produce the substrate for starch deposition. Therefore, altering the source/sink relationship in maturing crop plants may modify both the yield and percentage protein in the grain. However, extending the capacity of the plant to produce substrate (source unlimited) by extending the period of photosynthesis during senescence may increase the capacity of the grain to utilise this substrate only if the sink is limiting.

The source/sink relationships in plants exhibiting the 'stay green' trait are not clear. Because the chlorophyll and thylakoid proteins that are retained represent about 25% of the total nitrogen in a mature leaf (Evans, 1988), a significant proportion of leaf protein N

is immobilised in the old, non-photosynthetic leaves of type C 'stay green' mutant plants. Indeed, higher levels of total nitrogen were found in senescent leaves of a 'stay green' *Festuca* mutant than in wild type plants (Hauck *et al.*, 1997). Furthermore, it was shown that in *Lolium perenne* the 'stay green' trait had significant consequences for plant nitrogen relations, with shoots showing higher 'sink strength' for recently absorbed N compared with those of normal plants. However, although the N-use efficiency might be expected to be lower in this class of 'stay green' mutants than in normal plants, there were no differences in the rates of dry matter production (Bakken *et al.*, 1997). Although a significant proportion of leaf protein N is immobilised in the old, non-photosynthetic leaves of type C 'stay green' plants, there are no differences in the rates of dry matter production (Bakken *et al.*, 1997).

When the work described in this thesis was begun, the effects of extension of photosynthesis on yield production in wheat were not known. The principal aim of this project was therefore, to develop a type of 'stay green' durum wheat that could be exploited in breeding programmes in order to increase the grain yield in this important crop.

We were able to identify some mutants with delayed leaf senescence by screening a population mutagenised with EMS. Initial screening was carried out in the field by visual evaluation of the degree of leaf yellowing resulting from chlorophyll loss, and four mutants were chosen which had similar timing of flowering but later timing of senescence.

In vitro experiments showed a marked delay in chlorophyll breakdown in the leaves of the mutants plants compared with those of the control plants.

However, although the loss of chlorophyll and the concomitant yellowing of the leaves are convenient and distinctive indicators of leaf senescence, this phenotype can be uncoupled from 'functional' leaf senescence (Thomas and Smart, 1993). Characterisation of the mutants was therefore performed by determining, *in vivo,* three parameters related to photosynthesis: chlorophyll content, net photosynthesis and the efficiency of the PSII (F_v/F_m) of the chloroplast.

The changes in these parameters observed from flowering until full senescence, display different kinetics when comparing parental and control plants. Indeed, extensions of the rate of net photosynthesis, chlorophyll content and photosystem II efficiency (F_v/F_m) were evident in all the mutants analysed.

The drastic decrease in the chlorophyll content of the flag leaves indicated that the senescence process started at least one week earlier in the control plants than in the mutants. The marked early decrease in net photosynthesis may indicate that photosynthesis and chlorophyll content are not necessarily coupled during the senescence of durum wheat but may be controlled by independent regulatory mechanisms.

The maximum efficiency of PS II photochemistry, measured as F*v*/F*m*, showed no significant changes until the onset of senescence in mutants plants. Furthermore, the extended high rate of photosynthesis and retention of chlorophyll in the leaves of mutants plants were accompained by high efficiency of PS II photochemistry. However, after the onset of senescence, the decreases in all the parameters analysed proceeded as in the control plants. The fact that the mutation(s) affect all the photosynthetic parameters indicates, therefore, that the mutation(s) are 'functionally stay green' mutants according to Thomas (Thomas and Smart, 1993), and are not just delayed in the loss of chlorophyll content.

Since the 'stay green' phenotype in durum wheat continues to photosynthesise for longer than normal, it might be expect to result in higher yield of crops. An increase of seed weight was indeed observed in all the mutants, demonstrating that the 'stay green' trait may be of value for increasing the yield of crops (Thomas and Smart, 1993).

An extension of photosynthesis may increase the photoassimilate traslocated to the sink, which could result in increased starch synthesis and hence increased seed weight in the mutants. However, because the protein percentage (often referred as protein content) is dependent on the amounts of both protein and starch (Jenner *et al.*, 1991) an increase in the starch content of the seeds may explain the observed decrease in total nitrogen.

Futhermore, less nitrogen was present in the albumin/globulin fraction. This fraction, mainly constituted by enzymes, is concentrated in the embryo which was smaller in the mutants.

In conclusion, altering the source in durum wheat by extending the green leaf area and therefore the duration of photosynthesis results in an increase in the yield and a decrease in the nitrogen content of the seeds. However, the amount of nitrogen in storage proteins is apparently unaffected by the duration of green leaf area.

Furthermore, sink limitation seems to exist in durum wheat. Therefore, transferring the 'stay green' trait (by breeding programmes) to commercial lines in order to increase the sink (e.g. by increasing the number of seeds per ear) is a worthwhile goal to achieve a better distribution of photoassimilate **(Fig. 5.1.)**.

The 'stay green' trait has been transferred to our best commercial varieties of durum wheat for growth in Italy and field experiments on yield are being carried out on the F_4 population. Furthermore, we are focusing on the effects of the mutation(s) on yield under drought stress condition, allowing us to determine the effectiveness of the 'stay green' trait on drought tolerance in durum wheat.

Extending leaf area duration is a worthwhile target in order to increase the duration of crop photosynthesis and hence the yield in durum wheat. The traditional genetic approach and the new molecular approaches may therefore be useful to identify the key regulators of the 'stay green' trait in durum wheat.

In recent years, continuing efforts have been made to identify genes associated with or induced during leaf senescence. A picture is emerging of the conditions that initiate leaf senescence and of the promoter elements required for SAG expression. However, little is known of the regulatory genes that coordinate senescence at the molecular level. Studies of mutants from several species have revealed genes that regulate some aspects of leaf senescence. In soybean and the grass *Festuca pratensis*, mutants have been identified that remain green long after wild-type leaves became yellow. However, this 'stay green' phenotype often results from a block in chlorophyll degradation and the mutants undergo

most of the other biochemical changes associated with leaf senescence. The mutations do not affect the regulation of senescence as whole.

In order to isolate genes whose expression is up or down regulated by the mutation(s), a comparative analysis between mutant and control plants was performed by differential display of mRNA. Differential display is a powerful tool for analysing altered gene expression. However, among the putative differentially expressed clones which are isolated, a high proportion usually prove to be false positive which represents a real obstacle to exploiting the full potential of the method. Therefore, I used two approaches to identy and discard the majority of false positives prior to cloning. Genes related to photosynthesis and senescence were cloned either by differential display or by screening a cDNA library, and their expression was determined by northern analysis of flag leaves of both mutant and control plants. Genes encoding the soluble form of starch synthase, glycine decarboxylase (P-protein), rubisco activase and glyceraldehyde 3-P dehydrogenase showed significantly higher levels of expression in leaves of mutant lines compared with those of control plants. However, the higher expression levels of photosynthesis - related genes in mutants plants is probably a consequence (i.e. pleiotropic effect) of the extension of green leaf area which is sustained by functional photosynthesis. Leaf senescence is generally associated with decreases in chlorophyll, proteins and nucleic acids, with the degradation of chlorophyll being the most obvious of these changes. However, senescence is well underway by the time the leaf is senescing visibly. Indeed, the earliest and the most significant change in cell structure is the breakdown of the chloroplast, the organelle that contains up to 70% of the leaf protein (Matile *et al.*,1992, Gan and Amasino, 1997). These changes coincide with the conversion of the leaf from a photosynthetically active tissue to a source of nutrients that are recycled through salvage pathways.

In leaves of control plants the chlorophyll concentration was 80% of the initial content and therefore, it is not surprising that the abundances of transcripts encoding proteins involved in photosynthesis decrease sharply during senescence.

Unlike the expression of the photosynthesis-related genes, the expression of other genes such as SAGW1 and SAGW2 is up-regulated during senescence, showing enhanced

expression in leaves of control plants. The functions of these gene can be predicted by sequence comparisons but their real role is not known. However, because transcripts from these genes are less detectable in mutants plants, they may play a role in delaying the rate of progress of the senescence process.

Further analyses are required of genes such as W2 (*myb* trancriptional factor), W1, SAWG12 and SAWG14 that shows no sequence similarities to previously sequenced genes in the databases in order to determine their functions.

In conclusion, the extended time of expression of photosynthesis-related genes and the lower expression levels of senescence-related genes confirm, at the molecular level, that the novel mutants have the characteristics of functional 'stay green' according to Thomas and Smart (1993). However, it should be noted the leaf senescence is only delayed in the mutants, but ultimately does occur. It has been postulated that there may be multiple pathways that respond to a wide range of factors, and that these pathways may be interconnected to form a regulatory network to control leaf senescence in *Arabidopsis*. This feature has been referred as the plasticity of leaf senescence (Gan and Amasino, 1997). Recently, a putative leaf senescence regulatory network has been demonstrated in *Arabidopsis* (He *et al.*, 2001). The authors suggested that blocking a particular pathway may not have a significant effect on the progression of senescence. However, except for a few examples (Wilkinson *et al.*, 1995; Schaller and Bleecker, 1995; Hajouj *et al.*, 2000), we do not know the nature of any of the key genes that regulate the initiation or progression of leaf senescence. Furthermore, the majority of SAGs identified so far are involved in the degradation and recycling of cellular components and are regulated by senescence-promoting factors such as ABA, brassinosteroids, dehydration and dark treatment. Therefore, it is predictable that blocking a particular gene of the network may not have a significant effect on the onset and the progression of senescence. However, blocking a regulatory gene, especially a gene that controls developmental age-dependent senescence, might have a drastic effect on the onset and the progression of senescence, giving rise to a functional 'stay green' phenotype.

Although we do not have information on the molecular nature of the gene(s) for the 'stay green' mutation(s) in durum wheat, the physiological and molecular studies reported here

suggest that they encode key regulatory components controlling functional leaf senescence rather than components involved in the degradation and recycling of cellular components (Nam, 1997; Smart, 1994).

Future work should include the analysis of both photosynthesis and senescence-related genes. These genes will be used as probes to compare, by northern analyses, their expression patterns during the development and senescence of both mutants and control plants. In addition it will be interesting to sequence the remaning genes that were differentially expressed (about 20 clones) and to search for additional genes that are differentially expressed in the mutants

A genetic approach using recombinant imbred lines (RIL) will also be performed in order to map the mutant genes.

Studies of the genetic and molecular bases of variability in sink/source relations in cereals can also be made using 'stay green' plants providing information on the biochemical and molecular basis for the extreme genetic variability in sink/source relations observed in different cereals.

Many species exhibit a progressive decline in the rate of photosynthesis after full leaf expansion, and it has been suggested that photosynthesis is feedback regulated by the accumulation of carbohydrates in source leaves (Wingler *et al.*, 1998). Senescence is also thought to be regulated by sugars (Hensel *et al.*, 1993). Given the possible interplay between photosynthesis and the initiation of senescence, it would be interesting to identify the signalling molecules that relate photosynthesis to senescence. Because sugars are primary products of photosynthesis, sugar levels could form part of the signalling system.

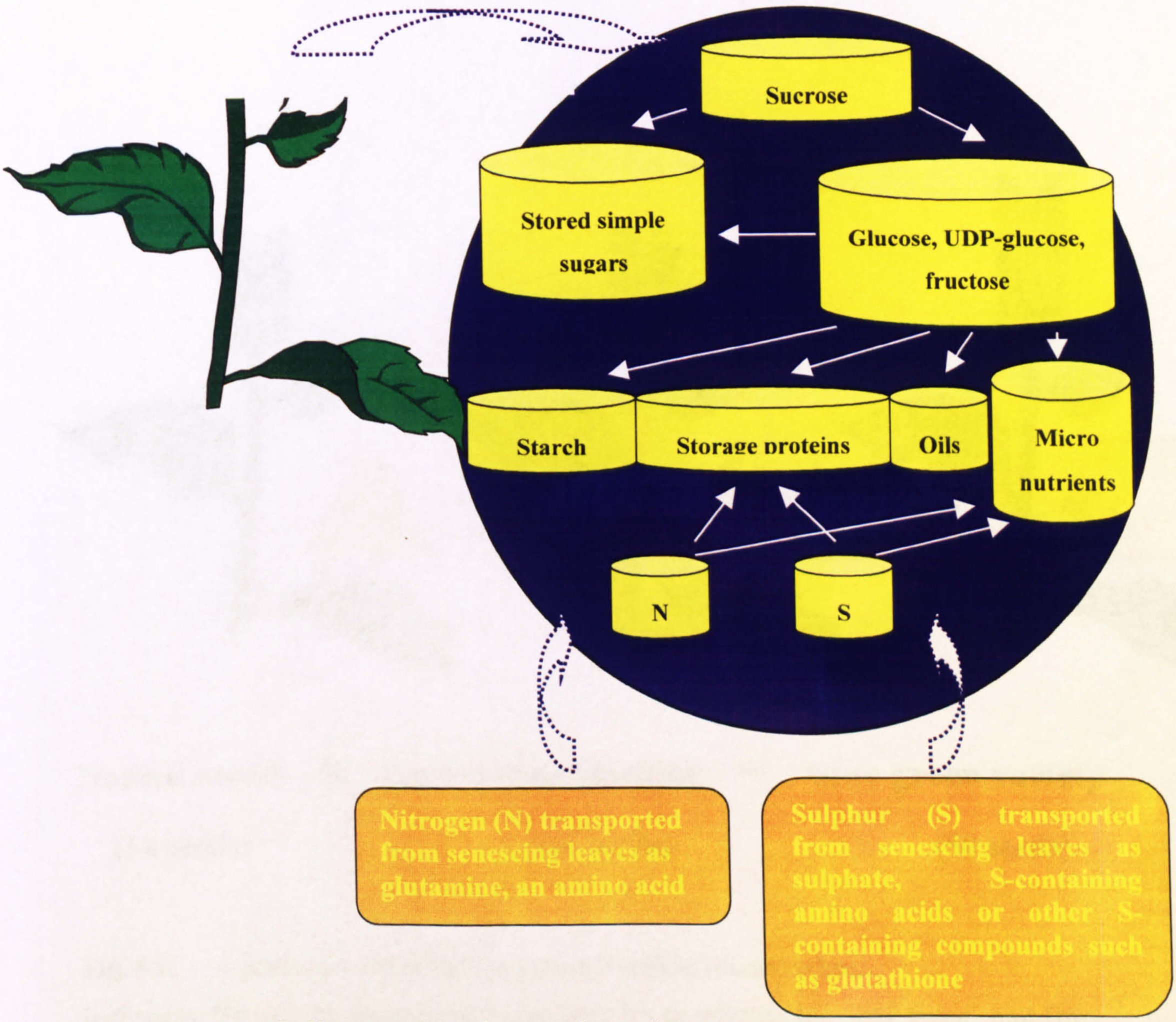


Fig. 5. Assimilate partition in a seed or tuber.

Carbon, nitrogen and sulphur are imported from photosynthesising and senescing leaves and from roots and are used to make a range of products (adapted from N.G. Halford, 1999).

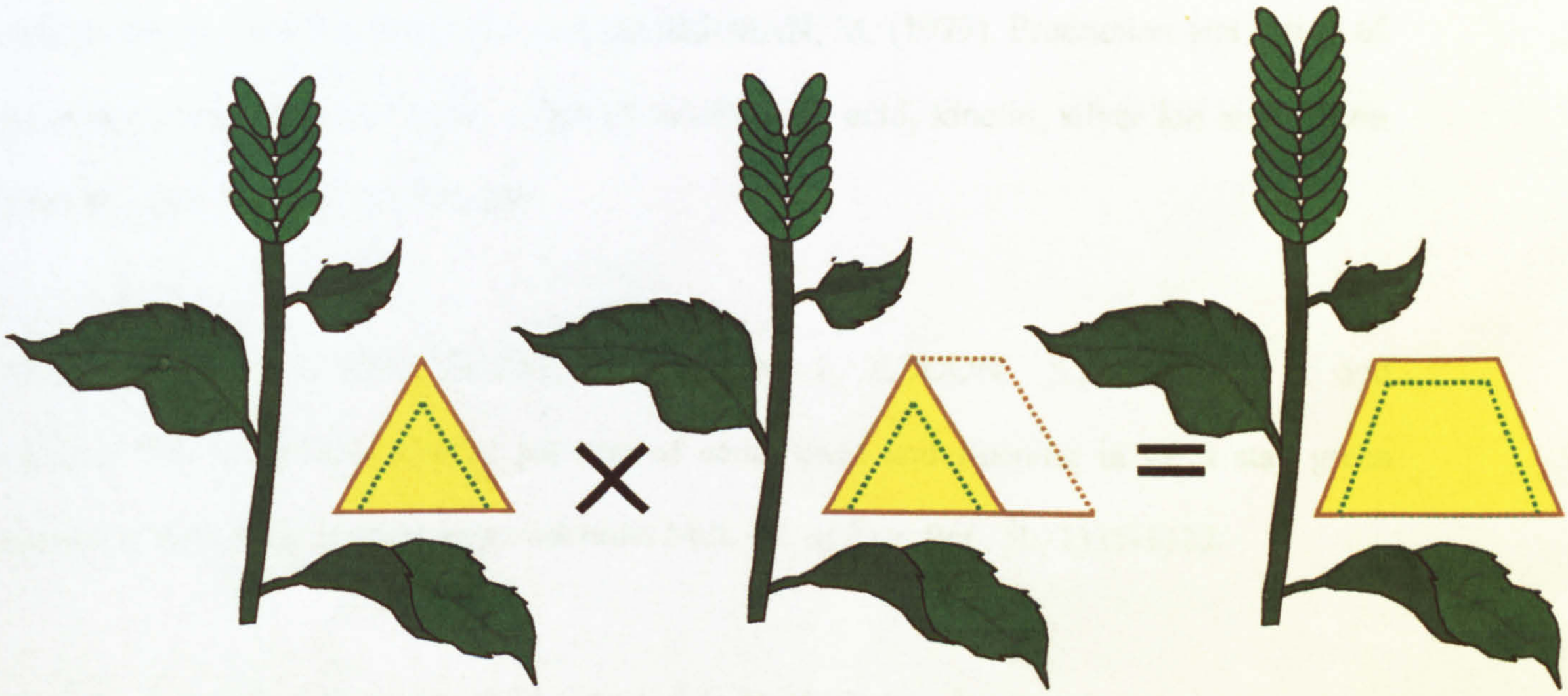


Fig. 5.1. A possible method to increase the sink in durum wheat.

Increasing the sink in wheat may be possible by transfering the 'stay green' trait (by breeding programmes) to commercial lines with more seeds per ear.

The aim is to obtained a wheat variety with a 'stay green' source and increase number of sinks.

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